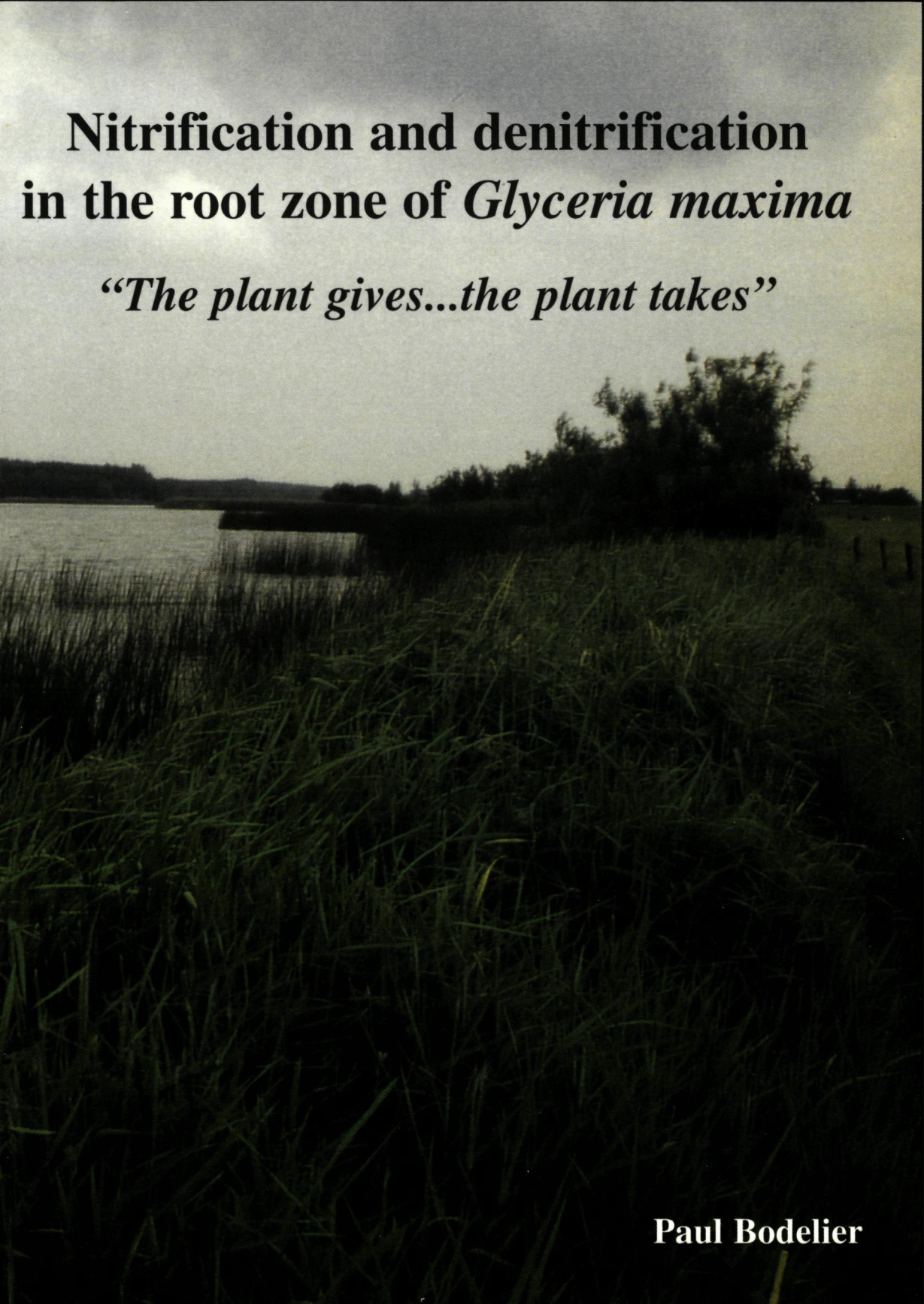
Nitrification and denitrification in the root zone of *Glyceria maxima*

"The plant gives...the plant takes"



Paul Bodelier

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een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

Proefschrift

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aan de Katholieke Universiteit Nijmegen,
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Contents

Voorwoord

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Chapter 1

General introduction

General introduction

Motivation

Nitrogen is an essential element of all living organisms as a basic component of proteins and nucleic acids. The availability and form of nitrogen in ecosystems is determined to a great extent by the microbiological processes as is presented in Figure 1.

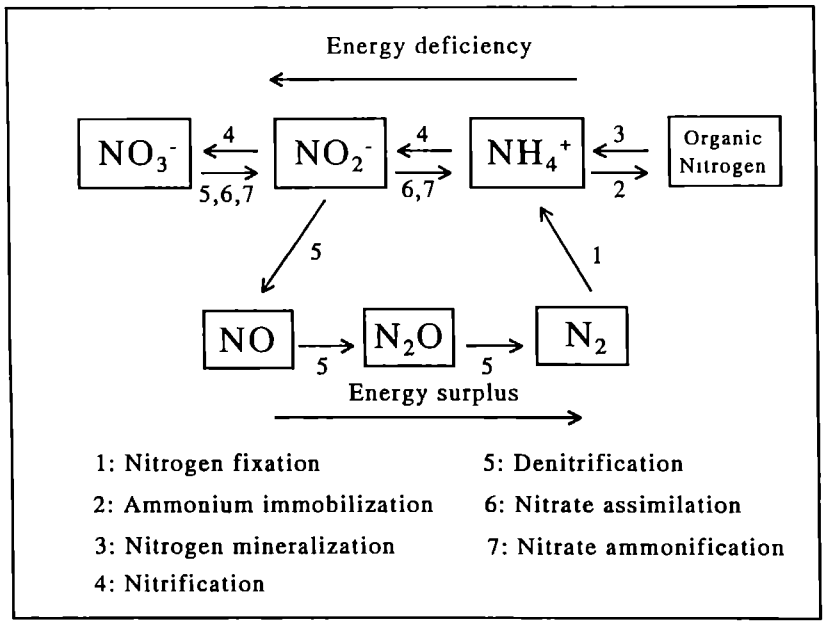


Figure 1 Schematic presentation of the cyclic nitrogen transformations as they occur in natural ecosystems (Redrawn from Woldendorp, 1981) In soils and sediments these processes are mainly driven by the input of energy in the form of organic compounds derived from plants When there is a sufficient supply of energy-rich organic compounds, nitrogen will be found in its reduced state as organic nitrogen, due to predominance of the energy-consuming steps of nitrogen fixation, nitrogen assimilation and denitrification When there is a shortage of organic compounds, oxidised nitrogen compounds will be formed due to the predominance of nitrification By excessive input of one of the nitrogen species an imbalance between these processes can occur

In natural ecosystems, these processes tend to balance each other (Woldendorp, 1981) but anthropogenic activities, which stimulate nitrification, can disturb this balance. Excessive nitrogen input due to industrial and agricultural activities have

lead to a surplus of reduced N-compounds resulting in a stimulation of nitrification which brings with it excess nitrate production and acidification of poorly buffered neutral soils (van Breemen and van Dijk, 1988, Stams *et al* , 1991) In addition, nitrate leaching from soils can give rise to eutrophication of surface waters (Roelofs, 1983) and elevated levels of nitrate in sub-soil water used for human and animal consumption Furthermore, both the elevated nitrification and subsequent denitrification in soils and sediments can result in higher emission of N_2O and NO to the atmosphere where it may act as a greenhouse gas or affect the ozone layer (Crutzen, 1981)
Besides the impact of nitrification and denitrification in environmental issues these processes are the prime regulators of nitrogen availability for plants Although, innumerable ecological and agricultural studies on these processes have been performed, the available information on the bacteria involved in nitrification and denitrification and the way they function and interact in soils and sediments is far from complete This lack of knowledge is most pronounced in flooded soils and sediments, where oxygen-releasing roots create oxic/anoxic interfaces and thus niches for both groups of bacteria This thesis aims to contribute to the knowledge and understanding of nitrifying and denitrifying bacteria by studying them in the root zone of oxygen-releasing plants

Oxygen-releasing plants

Upon flooding oxygen transport to non-vegetated soil or sediment is completely dependent on diffusion from the flood water This process is $\pm 10^4$ times slower than O_2 diffusion from the atmosphere (Ponnamperuma, 1984) In combination with chemical as well with biological oxygen consuming processes, this restricted oxygen supply will result in oxygen depletion The absence of oxygen results in a shift from aerobic to anaerobic microbial processes, which leads to reduction of oxidised compounds (NO_3^-, NO_2^-, Fe^{3+}, Mn^{4+}, SO_4^{2-} and CO_2) The processes, and micro organisms involved, have been reviewed by Laanbroek (1990) A number of the anaerobic microbial reductions yield phytotoxic compounds, such as S^{2-}, Fe^{2+} and Mn^{2+} Due to these fluctuating anaerobic conditions and the aerobic respiratory metabolism of plant roots it becomes apparent that plant growth in irregular or permanently flooded soils or sediments must be facilitated by specific adaptations The various mechanisms plants have developed to survive flooding in different habitats have been the subject of a number of reviews (Drew, 1983, Armstrong *et al* , 1994, Blom *et al* ,1994, Blom and Voesenek, 1996) Changes in anatomy, morphology and metabolism are of paramount importance for surviving in anoxic root environments Furthermore, adaptations on the life-cycle level may be important to survive flooding (Voesenek, 1990, Van der Sman, 1992) However, the most common and important adaptation to survive long-term flooding is probably the development of a gas-space continuum, primarily in the cortical

tissue, which stretches from the stomata to the root-root cap junction, which is referred to as aerenchyma Aerenchymatous tissue is present in a vast array of plant species (Justin and Armstrong, 1987, Laan *et al* , 1989a) Aerenchymatous tissue can be formed lysigenously (lysis of cells in the cortex), or schizogenously (cell separation) (Smirnoff and Crawford, 1983) and reduces the diffusive resistance for gasses Therefore, oxygen, originating from the atmosphere or from photosynthesis can diffuse from the shoot to the root allowing aerobic root metabolism to proceed However, the root will also loose oxygen to the surrounding soil or sediment by means of radial oxygen loss (ROL) ROL occurs when the respiratory demand of the roots is lower than the supply from the shoot or when biological or chemical oxygen consuming processes in the rhizosphere are closer to the oxygen in the cortical tissue than the oxygen consuming root tissue (Armstrong *et al* , 1994) A "sensible" figure for the amount of oxygen released *in situ* cannot be given as this is not only species and even root specific, but also dependent on soil physical factors such as compaction (Engelaar *et al* , 1993) and redox potential (Kludze *et al* , 1993, Kludze and Delaune, 1996) Moreover, all available techniques to measure oxygen release have serious drawbacks and are performed under artificial conditions as has been argued by Sorrell and Armstrong (1994) Another complicating factor is that radial oxygen loss does not occur along the whole root system but is often restricted to the young parts and apices of adventitious and secondary roots, as was demonstrated for *Phragmites australis* (Cav) Trin, ex Steud (Armstrong and Armstrong, 1988), *Oryza sativa* L (Armstrong, 1971) and *Rumex* species (Laan *et al* , 1989b) The aerenchymatous tissue enables plants to maintain root respiration and by ROL the rhizosphere may become oxidised and accumulation of reduced phytotoxic compounds (Fe^{2+}, S^{2}, Mn^{2+}) will be low Mineral nutrients, may be oxidised and available for plant use, and thus the plant creates an niche for its own roots in otherwise adverse anoxic habitats

Radial oxygen loss and the rhizosphere

By introducing oxygen into flooded soils or sediments aerenchymatous plants create oxic/anoxic interfaces in otherwise reduced environments thus enabling the progress of nutrient cycles Reduced compounds like NH_4^+, Mn^{2+}, Fe^{2+}, S^{2} and CH_4 diffuse into the oxic rhizosphere and are subsequently used by bacteria as electron donor in the oxidation with oxygen as electron acceptor The oxidised products can be used again as electron acceptor by obligatory or facultatively anaerobic bacteria in the anoxic soil or sediment layers or can be used as nutrients by the plants (Laanbroek, 1990) Besides the effects on nutrient cycling and availability, ROL can also lead to a reduction of the emission of the greenhouse gas CH_4 from wetland environments, which has been estimated to account for 10-30% of the global CH_4 emission Several wetland plants have proved to provide

methanotrophic bacteria with oxygen to oxidise a substantial part of the produced CH_4 (Epp and Chanton, 1993, Gilbert and Frenzel, 1995) ROL can, however, facilitate emission of harmful compounds by promoting the oxidation of ammonia to nitrite and nitrate by nitrifying bacteria with the subsequent reduction of these compounds by denitrifiers as outlined in the first paragraph of this chapter Additionally, nitrifying bacteria are themselves known to contribute to NO and N_2O emission from soils and sediments under reduced oxygen tensions (Bremner and Blackmer, 1981, Kester, 1996, Kester *et al* , submitted) which prevail in the rhizosphere of aerenchymatous plants The plant-facilitated removal of ammonia by "coupled" nitrification-denitrification has been the topic of numerous studies which have emphasized on fertilizer loss from agro-ecosystems Most of these investigations deal with the nitrogen economy of rice soils (Reddy and Patrick, 1984, Reddy and Patrick, 1986, Keeney and Sahrawat, 1986) as nitrogen is the limiting nutrient in the growth of this crop which feeds 50% of the worlds population (Reddy and Patrick, 1984) The very inefficient use of nitrogen by rice (Vlek and Byrnes, 1986), makes fertilizer N susceptible to nitrification-denitrification loss from the system, leading to very uneconomic fertilizer use In contrast, wastewater treatment research aims to optimize coupled nitrification-denitrification for the efficient removal of ammonia from wastewater This is often performed using oxygen-releasing plants in constructed wetlands (Reddy *et al* ,1989a, Brix, 1994, White, 1995)

However, as already mentioned the nitrifiers have to share the plant-derived oxygen with a number of chemical as well as biological oxidation processes The oxidation of organic carbon compounds by heterotrophic bacteria will be an important oxygen-consuming process in the rhizosphere where carbon is supplied by the roots via exudation, secretion and cell lysates, which can be as high as 40% of the total plants dry matter production (Marschner and Romheld, 1996) Thus, under oxygen-limiting conditions the nitrifying bacteria will have to compete for oxygen with heterotrophs An important part of the heterotrophic community in soils and sediments is represented by denitrifying bacteria, among which species of the genus *Pseudomonas* are the most abundant (Gamble *et al* , 1977, Knowles, 1982, Zumft, 1992, Nijburg *et al* , 1997) These facultatively anaerobic bacteria can not only compete for oxygen with nitrifiers but can also use the nitrate/nitrite produced by nitrifiers as electron acceptor

The following sections will discuss the processes of nitrification and denitrification and the possible interactions between the involved organisms in the rhizosphere of oxygen-releasing plants

The nitrification process

General

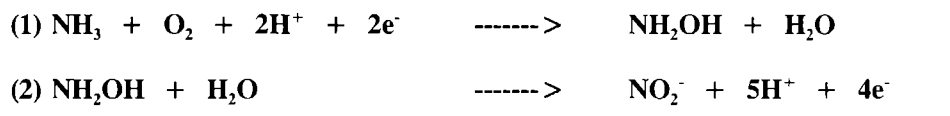
Nitrification is the conversion of the most reduced form of nitrogen (NH_3) to the most oxidised form (NO_3), and is mediated by bacteria belonging to the family of the *Nitrobacteriaceae* (Watson *et al* , 1989) These organisms are present in aquatic and terrestrial ecosystems and have a chemolitho-autotrophic metabolism, which means that they derive their energy from the oxidation of inorganic nitrogen compounds and can use CO_2 as sole carbon source for production of cell components (Wood, 1986) The oxidation of ammonia to nitrate is a two step process, performed by different organisms which are distinguished by their substrate utilisation The oxidation of ammonia to nitrite is performed by ammonia-oxidising bacteria, whereas the oxidation of nitrite to nitrate is performed by nitrite oxidising bacteria The characteristics of these organisms will briefly be described below Besides chemolitho-autotrophic nitrate production, many heterotrophic microorganisms are also able to produce nitrite or nitrate from reduced organic or inorganic nitrogen forms (Killham, 1986, Kuenen and Robertson, 1987) The closely related methane-oxidising bacteria can also oxidise ammonia to nitrite (Hanson and Hanson, 1996), but neither heterotrophic nitrifiers nor methane-oxidising microorganisms produce energy for growth by nitrification and have to date rarely been shown to contribute significantly to nitrate/nitrite production in natural soils or sediment, except for some acid soils This thesis will therefore only consider autotrophic nitrifying bacteria

Ammonia oxidisers

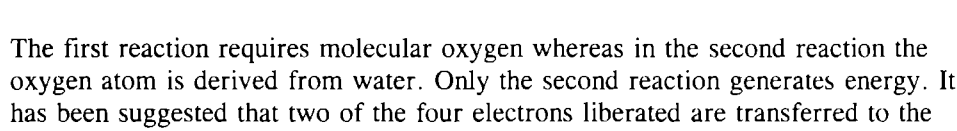
Ammonia oxidisers are placed in five genera on the basis of cell-shape, which is reflected in their names *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, and Nitrosovibrio* Phylogenetic analysis based upon 16S-rRNA sequence data places the ammonia-oxidising genera in the beta and gamma-subgroup of the proteobacteria (Teske *et al* , 1994), with only *Nitrosococcus oceanus* and *Nitrosococcus halophilus* falling into the latter category The genera *Nitrosospira, Nitrosolobus* and *Nitrosovibrio* have been classified as one genus, *Nitrosospira*, on the basis of the high similarity of the 16S-rRNA gene (Head *et al* , 1993) The most extensively studied ammonia oxidiser is *Nitrosomonas europaea*, as it has been the species most frequently isolated in pure culture (Prosser, 1989) However, culture (Belser, 1979) as well as molecular-based studies (Hiorns *et al* , 1995, Stephen *et al* , 1996, Kowalchuk *et al* , 1997) have shown that *Nitrosospira* species are common in many soils and sediments

Ammonia oxidisers are Gram-negative obligatory aerobic bacteria, which grow optimally at 25-30 °C, pH 7 5-8 0 and at ammonium concentrations from 2-10

mM. The generation time varies from 8 hours to several days (Bock *et al.*, 1986). The oxidation of ammonia proceeds in a two step reaction. Ammonia is oxidised to hydroxylamine by the enzyme ammonia monooxygenase (AMO), and hydroxylamine oxidoreductase (HAO) catalyses the oxidation of hydroxylamine to nitrite, as is presented by the following equations:



(1) $NH_3 + O_2 + 2H^+ + 2e^- \dashrightarrow NH_2OH + H_2O$



(2) $NH_2OH + H_2O \dashrightarrow NO_2^- + 5H^+ + 4e^-$

The first reaction requires molecular oxygen whereas in the second reaction the oxygen atom is derived from water. Only the second reaction generates energy. It has been suggested that two of the four electrons liberated are transferred to the terminal oxidase via cytochrome *c554* and cytochrome *c552* (Yamanaka, 1996). The other two electrons are transferred to the monooxygenase reaction via a reverse electron flow by the ubiquinone-cytochrome-b region of the electron transport chain and NAD^+, as suggested by Wood (1986). The flow of electrons in ammonia oxidation, however is yet to be demonstrated experimentally Ammonia monooxygenase, which is located in the cytoplasmic membrane, appears to be a versatile enzyme capable of oxidising a number of substrates including which methane (Bèdard and Knowles, 1989). The conversion of one mol of NH_3 to NO_2^- yields 273 kJ mol^{-1}, which is among the least favourable yields possible with oxygen-consuming redox-reactions. Ammonia-oxidising bacteria spent approximately 80% of the energy generated by this reaction on the incorporation of CO_2 into cell material by the Calvin cycle (Wood, 1986). Due to the lack of the enzyme α-ketoglutarate dehydrogenase, ammonia oxidisers have an incomplete tricarboxylic acid cycle which can only function biosynthetically and can not be used for energy generation as in heterotrophs (Schmidt, 1982). Assimilation of organic compounds by ammonia oxidisers has, however, been shown. Incorporation of small organic compounds like acetate and formate led to higher cell yields but was not related to energy generation (Krümmel and Harms, 1982). Despite their obligatory aerobic metabolism, ammonia-oxidising bacteria are able to reduce nitrite to NO and N_2O at low oxygen concentrations, with concurrent oxidation of ammonia to hydroxylamine (Poth and Focht, 1985). The amount of N_2O and NO produced in concert with the oxidation of ammonia can be as high as 10% in batch cultures (Goreau *et al.*, 1980). Production of N_2O and NO in soil and sediments due to ammonia oxidisers has also been demonstrated (e.g. Bremner and Blackmer, 1981; Kester *et al.*, 1996; Kester *et al.*, submitted). Nevertheless, an energy-generating function of NO and N_2O production has not yet been found and the ecological relevance of this reaction for the ammonia oxidisers is still not clear.

Nitrite oxidisers

The classification of the 4 genera of nitrite oxidisers, *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira* have been determined according to cell shape and ultrastructure Phylogenetically the known nitrite-oxidising genera have been affiliated to the alpha, delta and gamma sub-groups of the proteobacteria (Teske *et al* , 1994) and phylogenetically are not closely related to the ammonia oxidisers Teske *et al* (1994) suggested that ammonia- and nitrite-oxidising bacteria have evolved independently out of photosynthetic ancestors Nitrite oxidisers are Gram-negative bacteria which grow litho-autotrophically with nitrite as energy source and carbon dioxide as main carbon source Species of the genus *Nitrobacter*, which is almost exclusively isolated from soil, can also grow mixo- and or heterotrophically Heterotrophic growth on acetate, caseine hydrolysate, glycerol or pyruvate has been demonstrated with *Nitrobacter agilis* (Bock, 1976, Kalthoff *et al* , 1979), with longer generation times than "conventional heterotrophs" Under mixotrophic conditions (nitrite and organic carbon compounds are simultaneously used) *Nitrobacter hamburgensis* shows higher growth rates when compared to autotrophic or heterotrophic growth of this species (Bock *et al* , 1983) Most species grow optimally at 25-30 °C, at pH 7 5-8 0 and at nitrite concentrations ranging from 2-30 *mM* The generation time varies from 10 hours to several days (Bock *et al* , 1986)
The nitrite oxidation is catalysed by the enzyme nitrite-oxidoreductase, with the oxygen atom supplied by H_2O and without detectable intermediates as presented in the following reaction

$$NO_2 + H_2O \text{ ------> } NO_3 + 2H^+ + 2e$$

Although molecular oxygen is required in the terminal oxidase step as electron acceptor, nitrite-oxidising bacteria are not strict aerobes Several *Nitrobacter* strains are capable of dissimilatory nitrate reduction in the absence of oxygen, producing nitrite, ammonia and nitrogen gasses (Freitag *et al* , 1987, Bock *et al* , 1988) From *Nitrobacter vulgaris* a nitrate reductase has been purified which produced NO (Ahlers *et al* , 1990) Energy gained from anaerobic metabolism can be stored as poly-hydroxy-butyrate (PHB) by *Nitrobacter* whereas this does not occur with chemolitho-autotrophically growing cells (Bock *et al* 1988)
In comparison to ammonia oxidisers, nitrite oxidisers are metabolically more versatile The fact that they can grow heterotrophically, mixotrophically and anaerobically may be important for survival in habitats or conditions not suitable for chemolithotrophic growth This is often used to explain the higher numbers of nitrite-oxidising bacteria found in soil as compared to ammonia oxidisers (Woldendorp and Laanbroek, 1989)

Regulating factors of nitrification

The most important factors regulating nitrification are the availability of NH_3, O_2, CO_2, the pH and temperature, each of which are extensively discussed by Focht and Verstraete (1977) and Schmidt (1982) In the context of this thesis the pH will be of minor importance since the pH of flooded mineral soils is mostly between 6 7-7 2, regardless of the pH of the drained situation (Ponnamperuma, 1978) Carbon dioxide has rarely been reported to be limiting for nitrification In the context of this thesis it will be of minor importance since CO_2 will not be limiting in the vicinity of respiring roots In flooded soils and sediments inhabited by oxygen-releasing plants, NH_3 and O_2 availability will be the key regulating factors for nitrifying bacteria Hence, plants roots, excreting oxygen and consuming ammonium will have a major impact on nitrification Therefore the discussion on the regulating factors will be continued in the paragraph "***Effects of oxygen-releasing plants on nitrification and denitrification***"

The denitrification process

General

Reduction of nitrate in anoxic environments is dominated by two dissimilatory processes respiratory denitrification and dissimilatory nitrate reduction to ammonium or nitrate ammonification Denitrification is the bacterial respiratory process that couples electron transport phosphorylation to the stepwise, sequential reduction of nitrogenous oxides Dissimilatory nitrate reduction to ammonium is used by a number of (facultatively) fermentative bacteria as a sink for electrons Both processes are distinguished from the assimilatory nitrate reduction by the fact that the oxidised nitrogen is not primarily used by the cell for biomass production, but serves as electron acceptor for energy generation The dissimilatory processes are inhibited by oxygen and therefore generally occur in anoxic habitats Denitrification has intensively been investigated and reviewed (Knowles, 1982, Kuenen and Robertson, 1987, Tiedje, 1988, Zumft, 1992) because of the important ecological, agricultural an applied aspects, and is the only dissimilatory process which falls within the scope of this thesis

Organisms involved

Denitrifiers are principally aerobic bacteria which have the ability to use nitrogen oxides as electron acceptor when oxygen becomes limiting This ability is common to a wide variety of physiological and taxonomical groups Almost 130 species within more than 50 genera have been shown to be involved in denitrification (Zumft, 1992) Half of the known species are members of the genera

Pseudomonas, Neisseria and *Bacillus* The most commonly found denitrifiers in soils are the genera *Pseudomonas* and *Alcaligenes* (Gamble *et al* , 1977), with *Pseudomonas fluorescens* being the most frequently isolated species Among soil isolates, denitrifiers comprise about 20% of the bacterial population capable of anaerobic growth, and 1-5% of the total heterotrophic population that can be isolated (Tiedje *et al* , 1982)
The energy sources of denitrifiers are predominantly of organic nature, but there are also genera which are able to grow lithotrophically or phototrophically The majority of the denitrifying organisms possess all the enzymes to reduce NO_3 to N_2, this is not, however, a general characteristic as some strains lack nitrate-reductase (nitrite dependent strains) or N_2O-reductase (N_2O as terminal product)

Denitrification pathway

The pathway of denitrification is generally thought to be

$$\underset{\downarrow}{NO_3} \text{ ----> } \underset{\downarrow}{NO_2} \text{ -----> } \underset{\downarrow}{NO} \text{ -----> } \underset{\downarrow}{N_2O} \text{ -----> } \underset{\downarrow}{N_2}$$
$$\quad\; \mathbf{NAR} \qquad\quad \mathbf{NIR} \qquad\quad \mathbf{NOR} \qquad\quad \mathbf{N_2OR}$$

The enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NO) and nitrous oxide reductase (N_2OR) catalyse these reactions, respectively Nitrite, nitric oxide and nitrous oxide have been demonstrated to be obligatory free intermediates, and the enzymes involved have been characterised as reviewed by Zumft (1992) and Ferguson (1994) Nitrate reduction does not always proceed to completion Intermediates can accumulate because of a lack of one ore more of the denitrification enzymes, as described in the previous section, or due to environmental conditions Whether N_2O or N_2 is the end product may depend on several factors High NO_3 or NO_2 availability, high O_2 concentration, low organic carbon availability, low pH, high S^2 and low temperature all can result in higher N_2O liberation in proportion to N_2 (Firestone and Davidson, 1989)

Factors controlling denitrification

Oxygen

In general the most important regulating factors for denitrification in soils and sediments are O_2 and the availability of NO_3 and organic carbon compounds Anoxic or sub-oxic conditions are general prerequisites for denitrification O_2 can prevent the expression of the genes for synthesis of the enzymes or may affect the enzymes themselves Competition for electrons between the electron transport chains for the reduction of nitrate and O_2, respectively, is another possible

inhibition mechanism for O_2 inhibition of denitrification Inactivation of denitrifying enzymes by oxygen has only been observed with the copper containing nitrite-reductase and N_2O reductase (Ferguson, 1994) Inhibition of gene expression seems to be the most important effect of oxygen on denitrification (Ferguson, 1994) The enzymes are not expressed at the same time but sequentionally in response to lowered oxygen concentration (Korner and Zumft, 1989, Coyne and Tiedje, 1990) It has been suggested that the regulation of enzyme expression by oxygen is controlled by the NO_3/NO_2 antiporter system by an oxygen-sensing protein similar to that of the FNR (fumarate-nitrate-reductase) system of *E coli* (Ferguson, 1994, Guest, 1995) which activates the nitrate-reducing enzymes and or the nitrate transport system The inhibition of nitrate uptake by denitrifiers due to oxygen has already been demonstrated by Hernandez and Row (1988)
Absolute values for general inhibitory oxygen concentrations for denitrification can only be arbitrarly due to the enormous variety of organisms, environmental growth conditions and complexity of the nitrate-reducing system A value of approximately 10 μmol O_2 l^{-1} (appr 4% of air saturation at 25°C) is, however, accepted as a value above which denitrification is inhibited (Tiedje, 1988) In aquatic environments, N_2O and NO_2 accumulate between 6 25-31 25 μmol O_2 l^{-1} (2 5-12 5% of air saturation) and complete denitrification occurs below 6 25 μmol O_2 l^{-1} (Downes, 1988) With a number of organisms, denitrification even at air saturation levels has been demonstrated This phenomenon of aerobic denitrification, or co-respiration of oxygen and nitrate, was found in batch cultures of *Thiosphaera pantotropha*, *Alcaligenes faecalis* and *Pseudomonas denitrificans* irrespective of oxygen concentration (Robertson *et al* , 1995) and in continuous cultures of *Comamonas sp* (Patureau *et al* , 1996) The occurrence of aerobic denitrification has only been observed in culture, and its relevance in soil or sediment is still not supported by experimental evidence

Nitrogen oxides

The availability of nitrate will regulate denitrification when oxygen is low and organic carbon is in excess, such as occurs in freshwater sediments or in sewage sludge (Firestone and Davidson, 1989) In soils the absence or presence of oxygen will be of primary importance Only when oxygen is not inhibiting denitrification and nitrate concentration is high, organic carbon may regulate denitrification The presence of nitrate, plus sub-oxic conditions, is necessary for the induction of expression of denitrifying enzymes (Ferguson, 1994) Nitrate induced full expression of nitrate, nitrite, nitric and nitrous oxide reductase at low oxygen concentrations (Korner and Zumft, 1989) Nitrite also functioned as inducer for especially the nitrite and nitric oxide reductases Hence, apart form functioning as electron acceptor, the nitrogenous oxides seem to be important in the activation of the denitrification enzyme synthesis

Organic carbon

Organic carbon is necessary as donor for nitrate reduction. Thus denitrification will be strongly correlated with the availability of water-extractable carbon compounds. The nature of these compounds (i.e. sugar, amino acid) can also influence denitrification (Woldendorp, 1963). Although the availability of suitable carbon substrates for denitrification is influenced by a vast number of organisms and processes, the most important is probably the presence of plants which introduce carbon into soils or sediments. This can occur by sloughed-off and lysed root cells, mucilage and exudation of low molecular weight compounds such as sugars, organic acids and amino acids. These plant-induced factors will be addressed below in further detail.

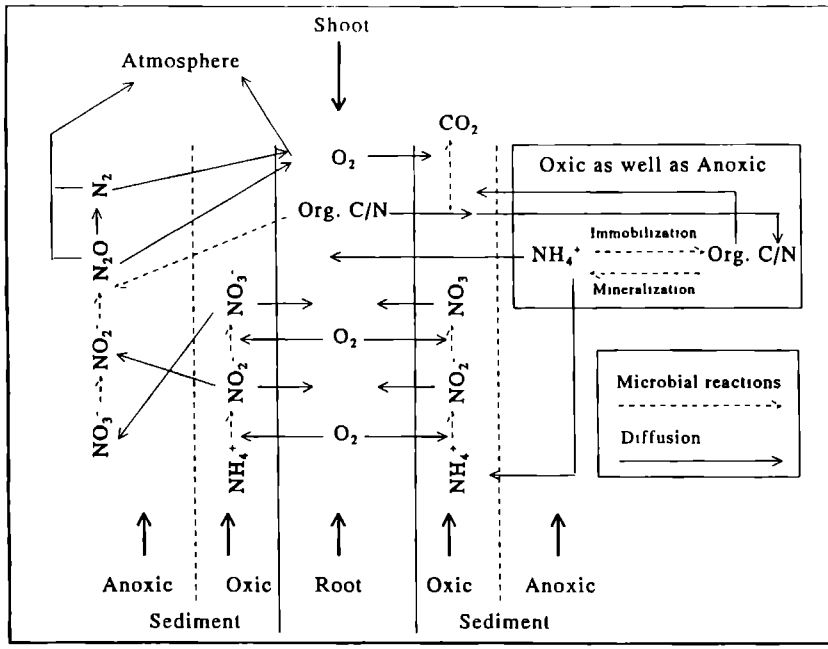


Figure 2 Schematic presentation of the possible effects of an oxygen-releasing root on nitrification and denitrification Oxygen which diffuses into the rhizosphere can be used by nitrifiers to convert ammonium into nitrate and by heterotrophs to oxidize organic carbon derived from the plant to CO_2 The nitrate produced can diffuse into the anoxic sediment layers where it is denitrified with organic carbon from the plant as electron donor The produced N_2 or N_2O can escape to the atmosphere via the sediment or via the aerenchyma of the plant The plant can also inhibit nitrification and denitrification directly by taking up nitrate or ammonium or by stimulating ammonium-immobilising processes

The effects of oxygen-releasing plants on nitrification and denitrification

Nitrification

It may be expected that, oxygen-releasing plants can control the regulatory factors effecting nitrification and denitrification (i e O_2, NH_4^+, NO_3^-, organic carbon) in their root zones, as is schematically presented in Figure 2 Oxygen release may stimulate nitrification directly or indirectly by stimulating nitrogen mineralization processes In contrast the plant can also suppress nitrification by the exudation of organic carbon compounds, which stimulate heterotrophic oxygen consumption and, depending on the C/N ratio, ammonium immobilisation Denitrification can be stimulated indirectly by elevation of the nitrate production in the oxic rhizosphere and directly by the provision of electron donors Inhibition of denitrification as a consequence of the presence of the plant is possible due to oxygen excretion and nitrate/nitrite uptake by the plant Plant effects on nitrification and denitrification will strongly depend on the relative amounts of oxygen and organic carbon excreted by the plant, which vary with plant species and environmental conditions There is no information available on the *in situ* availability of oxygen and organic carbon in the root zone of oxygen-releasing plants because of the continuous consumption of substrates by bacteria and plants Thus it is still not possible to quantify these parameters without disrupting the natural system *In situ* oxygen release in sediments surrounding oxygen-releasing roots has been investigated with microelectrodes in case of the emergent macrophyte *O sativa* (Frenzel *et al* , 1992) and for the submerged macrophytes *Lobelia dortmanna* (Pedersen and Sand-Jensen, 1995), *Potamogeton perfoliatus* L and *Zostera marina* L (Caffrey and Kemp, 1991) These studies demonstrated light-induced, and thus diurnal, oxygen release of the submerged macrophytes, whereas oxygen release of rice depended on diffusion from the atmosphere These studies were not combined with measurements of nitrification and denitrification The only information on the effects of oxygen release on nitrification has been obtained indirectly The detection of nitrifiers in the root zone of oxygen-releasing plants has to date only been performed incidently Both *et al* (1992a) found two orders of magnitude higher numbers of ammonia- and nitrite-oxidising bacteria in the rhizosphere of the emergent macrophyte *Glyceria maxima* (Hartm) Holmb than in the reduced bulk sediment Uhel *et al* (1989) found equal numbers of ammonia oxidisers in planted and unplanted rice soil Engelaar *et al* (1991) observed a negative effect of the oxygen-releasing *Rumex palustris* on numbers and potential activities of ammonia oxidisers However, this was due to ammonia limitation caused by plant uptake, as was demonstrated by preservation of the nitrifying activity when sufficient ammonia was present (Engelaar *et al* 1995) Hansen and Ostergaard-Andersen (1981) found potential nitrifying activities in sediments inhabited by *P australis* which were 3 times the values found in bare sediments, but Wittgren (1988)

measured ammonia oxidation rates which were lower in field lysimeters planted with *G maxima* as compared to bare plots, probably due to ammonium uptake by the plant Other reports on direct observations of nitrifiers in the root zone of oxygen-releasing plants are not available

Elevated levels of nitrate reductase in the plant is considered to be an indication of nitrate uptake and thus of nitrate production in the rhizosphere Nitrification in flooded soils has been demonstrated by this method (Blacquière, 1986, Uhel *et al* , 1989, Engelaar *et al* , 1995)

Another indirect method to determine nitrification activity in the root zone is by measuring elevated denitrification activity (i e N_2O/N_2 emission from planted soils or sediments) due to nitrate produced in the root zone Plant-mediated nitrate production has been demonstrated in this way with the use of ^{15}N fertilizer (Reddy and Patrick, 1986, Reddy *et al* , 1989b, Mosier *et al* ,1990, Caffrey and Kemp, 1992) and with the addition of NH_4^+ or urea (Smith and Delaune, 1984, Lindau *et al* , 1990)

Root zone nitrification in sediments has also been detected by analysing pore water NO_3 concentrations, as demonstrated for the submerged macrophytes *Zostera marina* (Iizumi, 1980) and *Littorella uniflora* (L) Ascherson (Christensen and Sϕrensen, 1986, Olsen and Andersen, 1994) The absence of nitrification when plants are not releasing O_2 has been demonstrated with experiments using dead *Spartina alterniflora* which showed no N_2O and N_2 production upon addition of $(NH_4)_2SO_4$ (Lindau and Delaune, 1991)

Denitrification

In sediments inhabited by wetland plants denitrification rate is often controlled by the availability of nitrate (Seitzinger, 1994) Addition of nitrate often leads to elevated denitrification compared to ammonium-based fertilizer (Reddy and Patrick, 1989b, Lindau *et al* , 1990, Nijburg *et al* , 1997) As already indicated, the plant may stimulate nitrification by excreting oxygen, but the nitrate produced is probably not enough to "saturate" the denitrifying bacteria This is supported by studies using wetlands designed for purification of wastewater where the conversion of ammonia in the root zone is the limiting step in the removal of nitrogen via denitrification (White, 1995, Wittgren and Tobiason, 1995) Moreover, the denitrifiers have to compete with the plant for the available nitrate, although this has never been observed directly in relation to oxygen-releasing plants When nitrate is present, the carbon supply by the plant will be very important for denitrification This has been studied intensively in non-waterlogged soils, where a distinct stimulation of denitrification by plant derived carbon was found (e g Woldendorp, 1963, Stefanson, 1972, Garcia, 1975, Klemedtsson *et al* , 1987b) Experiments with flooded soils or sediments have concentrated mostly on the loss of nitrogen from the system, which has shown to be higher in planted vs unplanted

systems due to plant carbon input (Reddy and Patrick, 1986, Reddy *et al* , 1989b, Lindau *et al* , 1990, Weisner, 1994) The provision of carbon by the plant can be influenced by several factors Nutrient deficiency (e g K or P) can lead to higher carbon loss Prade and Trolldenier (1990) demonstrated higher denitrification with potassium deficient plants The photosynthetic activity of the plant has also been shown to affect denitrification A positive relation between photosynthesis and denitrification was found by Scaglia (1985) and Bakken (1987) which they ascribed to higher carbon loss by the plant These results imply that plants can have diurnal and seasonal effects on denitrification Christensen and Sørensen (1986) demonstrated a strong seasonal pattern of denitrification in the root zone of the submerged macrophyte *L uniflora* In nitrate amended cores, a stimulation of denitrification was only found in summer, when high root biomass and subsequent higher carbon loss were observed These authors also demonstrated a diurnal effect Denitrification in the lower root zone of nitrate amended cores was higher in the light than in the dark, indicating higher carbon loss during the daytime In the upper root zone, denitrification was inhibited during daylight hours, presumeably due to oxygen release by the plant Inhibition of denitrification due to oxygen release was also suggested by Prade and Trolldenier (1990) who found higher denitrification associated with waterlogged *Triticum aestivum* L in comparison to *O sativa* which releases more oxygen into the root zone
Thus oxygen-releasing plants can influence the overall denitrification process by introducing oxygen, releasing organic carbon and taking up nitrate However, plant effects can also be at the level of the composition of the nitrate reducing bacterial community in the root zone Brunel *et al* (1992) demonstrated that enrichment of rhizosphere sediment from *Typha angustifolia* in chemostats with either oxygen or nitrate led to dominance of denitrifying Pseudomonads over nitrate ammonifiers Nijburg *et al* (1997) found higher denitrification activities, higher numbers of denitrifiers and dominance of denitrifying bacteria (*Pseudomonas* and *Moraxella* species) in the root zone of *Glyceria maxima* enriched with nitrate Without nitrate addition the nitrate reducing community consisted almost entirely of nitrite-accumulating and nitrate-ammonifying bacteria Hence, by taking up nitrate and excreting oxygen and carbon the plant may promote certain members of the nitrate-reducing community
The previous sections clearly indicate that oxygen-releasing plants can provide and deprive nitrifying and denitrifying bacteria from their substrates ammonia, oxygen, nitrate and organic carbon It is evident that the nature of the interactions between both groups of organisms will strongly depend on substrate availability and thus on the plant Taking into account the vast number of organisms and processes which are possibly involved in consumption of substrates for nitrifying and denitrifying bacteria, it is evident that substrate-limiting conditions are predominant in the root zone Competition will therefore exist for available resources Thus, parameters addressing the competitive abilities of co-occurring organisms are important in the

analysis of interactions between nitrifiers and denitrifiers in the root zone of oxygen-releasing plants

Table 1 Parameters describing the substrate uptake abilities of nitrifiers and (denitrifying) heterotrophs as reported in literature

Parameter	NH_3-oxidisers	NO_2-oxidisers	Denitrifiers (O_2 e acc)	Denitrifiers (NO_3 e acc)
μ_{max} (h^{-1})	0 014-0 088[1]	0 018-0 058[1]	0 1-0 33[2]	0 07-0 31[3]
Yield (g dry weight mol^{-1})	0 4-1 4[1]	1 11-1 51[1]	34 5-144[4]	14-78[5]
K_m e donor (mM)	0 018-14[6]	0 036-3 6[7]	0 004-5 56[8]	-
K_m e acceptor (μM)	1-40[9]	10-166[10]	0 01-3 13[11] 0 018-6 5[12]	1 71-290[13]
V_{max} e donor	4-200[1] (nmol NO_2/NO_3 g^{-1}cells $^{-1}$) 2 5-31[15] (fmol NO_2 /NO_3 cell $^{-1}h^{-1}$)	15 7-25 2[1] 1-42[7]	0 59-2 45[14] (mmol O_2 g^{-1} cells h^{-1})	-
V_{max} e acceptor (mmol g^{-1}cells h^{-1})	7 4-29 6[16]	5-28 6[16]	2 87-3 14[14]	0 19-5 14[17]
Specific affinity e donor (l g^{-1}cells h^{-1})	2 08-17 5[16]	3 7-105 6[18]	137-471[14]	-
Specific affinity e acceptor (l g^{-1}cells h^{-1})	600-6500[16]	200-1300[16]	8971-15157[14]	111-117[17]

[1] Prosser 1989 [2] From Koike and Hattori (1975) and Robertson and Kuenen (1990) [3] From [2] and Rehr and Klemme (1989) [4] From Koike and Hattori (1975), Gerritse *et al* (1992), Sharma and Ahlert (1977) [5] Koike and Hattori (1975), Carlson (1983), Rehr and Klemme (1989) [6] Prosser (1989), Verhagen and Laanbroek (1991), Laanbroek and Gerards (1993), Laanbroek *et al* (1994) [7] See [6] and Both *et al* (1992b) [8] Sharma and Ahlert (1977), Button (1985), Gerritse *et al* (1992) [9] Sharma and Ahlert (1977), Goreau *et al* (1980), Stenstrom and Poduska (1980), Painter (1986), Laanbroek and Gerards (1993) and Laanbroek *et al* (1994) [10] Sharma and Ahlert (1977), Stenstrom and Poduska (1980), Laanbroek and Gerards (1993) and Laanbroek *et al* (1994) [11] Longmuir (1954), Sharma and Ahlert (1977), Gerritse *et al* (1992) [12] Poole (1983), values for bacterial cytochromes found in heterotrophs [13] From Knowles (1982), Christensen and Tiedje (1988) and Rehr and Klemme (1989) [14] Button (1985) and Gerritse *et al* (1992) Converted assuming a cell carbon content of 55% and 25% dry weight per cell [15] Prosser (1989), Laanbroek and Gerards (1993) and Laanbroek *et al* (1994) [16] Laanbroek and Gerards (1993) and Laanbroek *et al* (1994), per cell rates were calculated to cell weight basis using a cell weight of 5×10^{-13}g (Sharma and Ahlert, 1977) [17] Carlson (1983) and Christensen and Tiedje (1988) [18] See [16] and Both *et al* (1992b)

Interactions between nitrifiers and denitrifiers in the rhizosphere of oxygen-releasing plants.

Substrate uptake by bacteria can be desribed by Michaelis-Menten kinetics as presented in the equation

$$V = (V_{max} \times S)/(K_m + S)$$

where V = actual substrate uptake rate, V_{max} = maximum substrate uptake rate, K_m = half saturation constant of the uptake system, S = substrate concentration
The K_m is the concentration of the limiting substrate at which the uptake rate is half of the maximum and is also referred to as the affinity constant A low K_m enables the organism to be active at low substrate concentrations Healy (1980) argued that the substrate sequestering ability of bacteria can be described by the ratio V_{max}/K_m (specific affinity), taking also the maximum conversion capacity of the organism into account Beside the uptake kinetics, the efficiency and rate of biomass formation, reflected in the yield and maximum growth rate (μ_{max}), will be important in competition Experimental values for these parameters for nitrifying and (denitrifying) heterotrophic bacteria are summarised in Table 1
The presented values are derived from pure culture studies, determined using a variety of methods and growth conditions and therefore should not considered to be absolute values Nevertheless, it is obvious that nitrifying bacteria have less competitive potential than heterotrophic denitrifiers The maximum growth rates are lower, as biomass production due to the oxidation of ammonia and nitrite is up to two orders of magnitude lower than heterotrophic production Conversion rates of electron donors are higher for denitrifiers, leading to higher specific affinities Autotrophic nitrifiers also have higher half saturation constants for oxygen, leading to lower specific oxygen affinities, with the nitrite-oxidising bacteria being the least competitive organisms for oxygen Thus, it is quite paradoxical that chemolitho-autotrophic nitrifying bacteria are able to be active and persist in the rhizosphere of oxygen-releasing plants Heterotrophic denitrifiers should grow more efficiently on the substrates which are continuously supplied by the plant and oxidise them with oxygen which they can more efficiently acquire Moreover, the number of potentially oxygen consuming organisms is not only confined to denitrifiers, which makes the occurrence of nitrification in the root zone of oxygen-releasing plants even more puzzling
In addition to oxygen, nitrifiers will also have to compete for ammonium and nitrite, which can both be used by heterotrophic bacteria and plants It has been shown that nitrifying bacteria are outcompeted by plants (Engelaar *et al* , 1991, Verhagen *et al* , 1994) and heterotrophic bacteria for limiting amounts of ammonium (Verhagen and Laanbroek, 1991) These latter authors demonstrated

that when available C/N ratio's of the substrates exceed 10, all ammonium will be immobilised by heterotrophic bacteria. The nitrite-oxidising bacteria also have to compete for nitrite with denitrifiers. As this can only occur in the presence of oxygen, the denitrifiers should be able to reduce nitrite in the presence of oxygen, which has already been demonstrated. It is also tenable that nitrite oxidisers might profit from nitrite produced intermediately by denitrifiers, as has been suggested by Belser (1977).
Negative effects of nitrifiers on denitrifying bacteria are very unlikely taking the data of Table 1 into account. By consuming oxygen and by producing alternative electron acceptors nitrifiers can in fact stimulate denitrifying bacteria. However, this will not only depend on the ability of the nitrifiers to function in the root zone, but also on the possible inhibiting effects of the plant on denitrification.
It is evident that the nature of the interactions between these two important groups of organisms in the nitrogen cycle in the root zone of oxygen-releasing plants will primarily be determined by the characteristics of the plant and its environment. The qualitative, quantative, spatial and temporal aspects of substrate availability will directly be governed by the plant. The characteristics of and the 'strategies' displayed by the bacterial community will also be fundamental in addressing the subject at issue in this thesis.

Aims and Outline of the thesis

This thesis aims to contribute to the understanding of bacteria involved in nitrogen conversions and the way they interact in the root zone of oxygen-releasing plants. The persistence of nitrifiers in this habitat, despite their poor competitive abilities will be a main issue as will be the effects of nitrifiers and the plant on denitrifiers. The approach adopted involves experimental use of model organisms and model habitats while monitoring the characteristics of representative organisms to determine a "reference" for comparison with their behaviour in the natural habitat, which has also been under investigation.
In chapter 2, substrate uptake kinetics of a representative denitrifying bacterium *Pseudomonas chlororaphis*, have been determined under exactly the same experimental conditions as has used done for the ammonia oxidiser *Nitrosomonas europaea* (Laanbroek and Gerards, 1993; Laanbroek *et al*, 1994). This approach allows for comparison of substrate utilization kinetics, gaining information on the controlling factors in the root zone.
Chapters 3 and 4 deal with a gnotobiotic microcosm which was developed to study interactions of pure cultures of nitrifying and denitrifying bacteria as influenced by oxygen-releasing plants. Effects of the emergent macrophyte *Glyceria maxima* on the denitrifying bacterium *P. chlororaphis* were determined using the photoperiod of the plant as a natural variable to manipulate carbon availability in the root zone,

as described in chapter 3 The photoperiod of *G maxima* was also used to manipulate the interactions between the nitrifiers *N europaea* and *Nitrobacter winogradskyi* and the denitrifier *P chlororaphis* in the gnotobiotic microcosms The impact of *G maxima* on the interactions between nitrifiers and denitrifiers was studied by determining population dynamics in the physically separated "macro"-rhizosphere and non-root sediment of the microcosms

Population dynamics of nitrifying and denitrifying bacteria were determined during a growing season inside and outside the root zone of a natural *Glyceria maxima* stand at a freshwater lake location in the Netherlands, as described in chapter 5 This was done to get information about the impact of oxygen-releasing plants under natural conditions and to determine the influence of the growth cycle of the plant on nitrification and denitrification Furthermore, adaptation of ammonia-oxidising bacteria to sub-oxic or anoxic habitats was investigated

In chapter 6 the analysis of the ammonia-oxidising community, as reported in chapter 5, by means of molecular techniques (e g PCR, DGGE, probe-hybridization) is presented This was done to determine what type of ammonia-oxidisers are associated to oxygen-releasing plants and whether seasonal dynamics in plant growth and nitrogen conversion rates, and possible adaptations are reflected in the species composition of the ammonia-oxidising community present

In chapter 7 the main conclusions will be presented together with a synthesis of the obtained results in this thesis

Chapter 2

Oxygen uptake kinetics of *Pseudomonas chlororaphis* growing in glucose- or glutamate-limited continuous cultures

With H.J. Laanbroek

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Oxygen uptake kinetics of Pseudomonas chlororaphis grown in glucose- or glutamate-limited continuous cultures.

Abstract

Oxygen uptake and glucose and glutamate oxidation kinetics of the heterotrophic bacterium *Pseudomonas chlororaphis*, grown in glucose- or glutamate-limited cultures under oxygen-saturating or oxygen-limiting conditions, were determined K_m values for oxygen were 1 4-5 6 μM Only in the case of glucose were significantly lower K_m values and enhanced specific oxygen affinity (V_{max}/K_m) per cell found under oxygen-limiting conditions Both K_m and specific affinity values for glucose and glutamate oxidation were apparently affected by oxygen concentration, although a statistically significant enhancement of the oxidation kinetics was only found for glutamate Discussing the kinetic data found for *P chlororaphis* led to the conclusion that the outcome of competition for oxygen with *Nitrosomonas europaea* in the rhizosphere of oxygen-releasing macrophytes will primarily be determined by oxidation kinetics of the electron donor instead of the oxygen uptake kinetics of the respective organisms

Introduction

On the basis of literature values reported for oxygen uptake kinetics, heterotrophic bacteria should outcompete autotrophic ammonia oxidisers in oxygen-limited environments, such as the rhizosphere of oxygen-releasing plants. The ammonia oxidisers are proposed to be outcompeted because of their inferior oxygen consumption kinetics (Sharma and Ahlert, 1977; Prosser, 1989; van Niel, 1991). The fact that ammonia-oxidising bacteria can be active in the rhizosphere of oxygen-releasing plants (Bodelier *et al.*, 1996, Engelaar *et al.*, 1995) implies that an excessive amount of oxygen is available, that carbon is limiting the heterotrophic bacteria or that ammonia oxidisers compete successfully for oxygen by using more favourable oxygen uptake kinetic parameters than have been reported to date. Published half saturation constants for oxygen uptake by heterotrophic bacteria are in the range of 0.018-6.5 μM O_2 depending on the type of cytochrome oxidase present (Poole 1983), while values for ammonia oxidisers are reported in the range of 7-40 μM (Sharma and Ahlert, 1977; Stenstrom and Poduska, 1980; Prosser, 1989). To date, there is only one study presenting a lower K_m value (1.3μM), determined for the ammonia-oxidising species *Nitrosomonas europaea* grown in oxygen-limited continuous cultures (Laanbroek and Gerards, 1993).

Prediction of the competitive outcome on the basis of kinetic values presented in literature may be suspect because of the variety of methods used to measure uptake kinetic parameters and differences in growth conditions prior to kinetic measurements. The latter can result in the presence of different cytochromes with different kinetic properties (Poole, 1983). Moreover, it is more appropriate to

compare specific affinities, defined as the ratio V_{max}/K_m, to study competitiveness at low substrate concentrations (Healy, 1980)
The goal of this study was to determine oxygen uptake kinetics of the heterotrophic bacterium *Pseudomonas chlororaphis*, which is common in the rhizosphere of the oxygen-releasing macrophyte *Glyceria maxima* (Nijburg *et al* , 1997), and compare these kinetic values with those of the ammonia-oxidising bacterium *Nitrosomonas europaea* on the basis of specific affinity (e g V_{max}/K_m) *Nitrosomonas europaea* also appeared to be abundant in the root zone of *G maxima*, as demonstrated by a positive reaction to specific antibodies for *N europaea* in the most diluted positive tube of a MPN enumeration (Bodelier, unpublished results) The heterotrophic bacterium was grown under energy-limited conditions, as has been done for *N europaea* (Laanbroek and Gerards, 1993), with glucose or glutamate as substrate, both of which are representative root exudates (Curl and Truelove, 1986)

Materials and Methods

Organism and culture conditions

Pseudomonas chlororaphis ATCC 43928 (Christensen and Tiedje, 1988) was grown in continuous culture in a 1250 ml "Biostat M" fermenter (Braun, Melsungen, Germany) at a dilution rate of 0 015 h^{-1} (pH 7 5, 25°C, 300rpm) Duplicate chemostats were operated to reach steady states under excessive (80% air saturation, ≈214 μM O_2, maintained by sparging with air) and limited oxygen supply (oxygen diffusion from the headspace) with glucose or glutamate as electron donors and sole carbon source When the oxygen supply was reduced, 0% air saturation was maintained by the oxygen-consuming activity of the cells The medium contained per litre 330 mg $(NH_4)_2SO_4$, 100 mg KH_2PO_4, 40 mg $MgSO_4$ $7H_2O$, 20 mg $CaCl_2$, 500 mg NaCl, 396 mg glucose H_2O or 353 mg glutamic acid and 1 ml trace element solution (Verhagen and Laanbroek, 1991) The pH of the culture was maintained at pH 7 5 by automatic titration with 0 1 *M* NaOH Steady-state was assumed when optical density (660 nm), cell numbers and glucose/glutamate concentrations remained constant for 5 volume changes

Measurements of oxygen uptake and substrate oxidation kinetics

Oxygen uptake and substrate oxidation kinetics were determined as initial rate measurements in a closed chamber with a polarographic oxygen electrode with washed cell suspensions from steady state cultures, using a Biological Oxygen Monitor (Oxygen meter 781, Strathkelvin Instruments Glasgow, Scotland) according to Laanbroek and Gerards (1993) Oxygen uptake kinetics were determined as the tangent of oxygen concentration versus time plots at different oxygen concentrations of 1-230 μM at 28°C in the presence of excess glucose or

glutamate. Substrate oxidation kinetics were determined by measuring initial oxygen consumption rates at concentrations of glucose or glutamate of 10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0.005 *mM* and saturating oxygen concentrations (90-100% air saturation). Substrate uptake/oxidation curves obeyed Michaelis-Menten kinetics as is shown in Fig. 1. Maximal uptake/oxidation rates (V_{max}) and saturation constants (K_m) were estimated with the computer program Enzpack version 2.0 (P.A. Williams, Bangor, Wales) using the "direct linear" method of Eisenthal and Cornish-Bowden (1974)

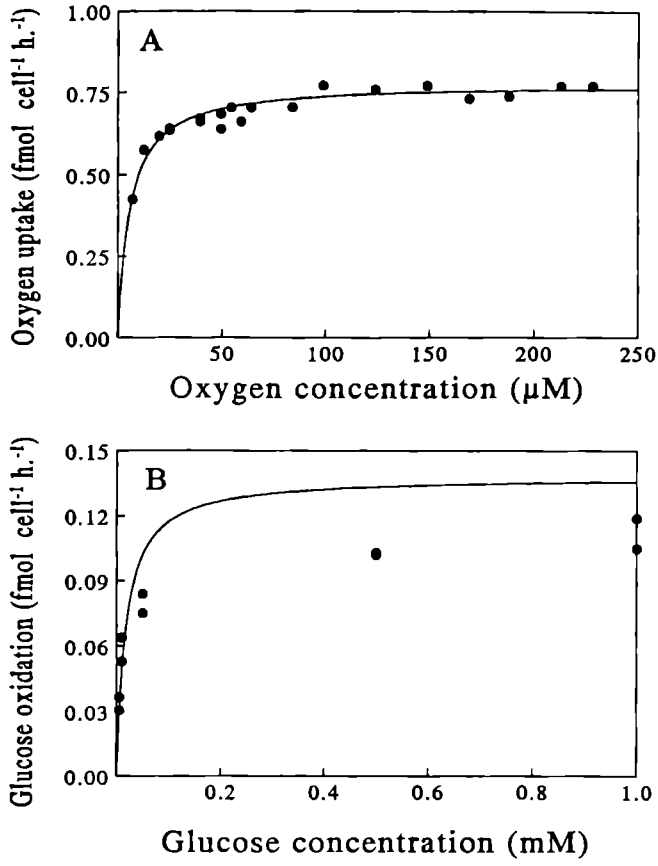

Figure 1 Typical oxygen uptake (A) and glucose (B) oxidation plots of washed steady state cell suspensions (8.4×10^8 ml^{-1}) of *P chlororaphis* as determined using a biological oxygen monitor The curves were drawn using the respective K_m and V_{max} values as estimated with the direct linear method Data fitted Michaelis-Menten equation, R^2 = 0 92 and 0 86 for oxygen uptake and glucose oxidation, respectively

Enumeration of P. chlororaphis cells

Cell counts were performed microscopically using a Burker-Turk counting chamber, immediately upon sampling of the chemostat Cell counts were also performed on the washed cell suspensions which were used for Biological Oxygen Monitor measurements

Analytical methods

Glucose concentration was measured with a glucose test combination (Boehringer Mannheim, Germany) based on the GOD-POD (glucose oxidase-peroxidase) method described by Werner *et al* (1970) Glutamate concentration was measured by means of a test combination (Boehringer Mannheim, Germany) based upon an enzymatic assay using glutamate dehydrogenase and diaphorase as described by Beutler (1985) Total, particulate and dissolved organic carbon were determined according to Verhagen and Laanbroek (1991), using a total organic carbon analyzer (model 700, O I Corporation, College Station, Texas, USA)

Results and Discussion

Cell numbers at 0% air saturation (0 36-0 72×10^9 ml ^{1}) were approximately 50% of those at oxygen saturation (0 96-1 3×10^9 ml ^{1}), indicating oxygen-limiting conditions Residual glucose concentrations during steady state were always below the detection limit (15-20μM) of the assay, while glutamate concentrations in the culture were 10μM at saturating oxygen conditions and 15μM at 0% of air saturation (data not shown) Besides the residual substrate- and biomass carbon, dissolved organic carbon in the cultures was relatively low (6-11% of the amount of carbon in the influent, data not shown) and independent of the oxygen concentration The reduced cell yield and the absence of accumulation of substrate or intermediates at low oxygen concentration, suggest uncoupling of energy generation and growth, as was demonstrated by Harrison and Loveless (1971), as the consequence of limited oxygen availability

The K_m values for oxygen found for *P chlororaphis* were in the range of those found for cytochrome *o* (1 8-6 5 μM) and cytochrome aa_3 (4-7 μM) (Table 1), cytochrome *o* being the most common cytochrome in bacteria (Poole 1983) With glucose as electron donor, specific oxygen affinity increased under oxygen-limiting conditions (Table 1), which was not the case with glutamate The expression of higher affinity cytochromes, such as cytochrome *d*, under oxygen limitation has been reported earlier (Poole 1983) However, K_m values for this type of cytochrome are 0 018-0 35 μM and thus much lower than the values in this study We compared oxygen kinetics of *P chlororaphis* with those of the autotrophic ammonia oxidiser *Nitrosomonas europaea*, using exactly the same methods and

growth conditions as reported previously (Laanbroek and Gerards 1993, Laanbroek *et al* 1994) Specific oxygen affinity for the ammonia oxidiser appeared to be higher, being 1 37 and 1 09 nl cell^{-1} h^{-1} for cultures grown at 80% and 0% air saturation, respectively (Laanbroek et al 1994) This difference was due to a substantially higher V_{max} for *N europaea*, which at oxygen-limiting conditions was 10 and 29 times the V_{max} for *P chlororaphis* with glucose or glutamate, respectively, as electron donor However, besides the oxygen uptake kinetics the yield has to be taken into account in predicting the outcome of the competition for oxygen between those organisms, as described by Button (1994) in the following equation

$$\mathbf{A = \mu/(a°_A \times Y)} \quad (1)$$

A = the substrate concentration required to reach growth rate μ given the specific substrate affinity ($a°_A$) and yield (Y) The product of specific affinity and yield determines the attainable growth rate at a certain substrate concentration At oxygen limitation, this product for oxygen as a substrate is higher for *P chlororaphis* for growth on glucose and glutamate (Table 1), compared to the same value for *N europaea*, which was 8720 l h^{-1} mol^{-1} (Laanbroek *et al* 1994) This difference is due to a lower yield value and not to inferior oxygen uptake kinetics of the ammonia oxidiser This implies that the critical oxygen concentration for *N europaea* at a dilution rate of 0 015h^{-1} in chemostats is higher than that of *P chlororaphis* leading to washout of the nitrifier before oxygen-limiting conditions are even reached

Assuming a situation where no washout takes place, as in soil, and a cell-to-cell competition for oxygen, one can calculate the oxygen concentration at which both organisms will acquire equal amounts of oxygen per cell, based on their K_m and V_{max} values The substrate consumption rate, V, would be equal for both organisms at this oxygen concentration (S) as indicated by the Monod equation in (2)

$$\mathbf{(V_{max1} \times S)/(K_{m1} + S) = (V_{max2} \times S)/(K_{m2} + S)} \quad (2)$$

Solving for substrate concentration S, produces equation 3

$$\mathbf{S = (V_{max1} \times K_{m2} - V_{max2} \times K_{m1})/(V_{max2} - V_{max1})} \quad (3)$$

Substitution of the V_{max} and K_m values of *P chlororaphis* and *N europaea* at 0% air saturation (D=0 015h^{-1}) in this equation with the measured values, produces S values -0 50 and -4 48μM O_2 for growth on glucose and glutamate, respectively Thus, at all oxygen concentrations, the oxygen consumption rate per cell is higher for *N europaea*, which must be regarded as the better competitor when no growth takes place

Competition only for oxygen is probably rare under natural conditions, as limiting amounts of the electron donors also influence the performance at low oxygen availability From the glucose and glutamate oxidation kinetics of *P chlororaphis* (Table 2) and data of Laanbroek *et al* (1994) it is evident that ammonia-oxidising bacteria have very poor electron donor kinetics In fact, the products of specific affinity and yield reveal that according to equation (1), the ammonium concentration for maintaining a growth rate of 0 015h^{-1} of *N europaea* have to be 40-70 times as high as the concentrations of glucose or glutamate for the growth of *P chlororaphis* This strongly suggest that a prerequisite for actual oxygen competition between ammonia oxidisers and heterotrophs is that ammonium is available in excess compared to the electron donor concentration for the heterotroph

Since ammonia-oxidising bacteria are not good in the acquisition of limiting amounts of ammonium and lose in competitions for this compound with plants and heterotrophic bacteria (Verhagen *et al* 1994, Verhagen and Laanbroek 1991), we can conclude that in most natural ecosystems it is more likely that ammonia-oxidising bacteria will be limited by ammonium than by oxygen

Table 1 Kinetics of oxygen uptake by *Pseudomonas chlororaphis*, grown in continuous culture (D=0 015h^{-1}, 25°C, pH 7 5) at 80% and 0% air saturation and with glucose or glutamate as electron donor Yields and product of specific affinity and yield are also presented Values are the means (±1SE) of two replicate chemostats Significant differences between means of the different parameters for oxygen are indicated by different letters (Tukey s test, $p<0$ 05, n=2)

Substrate uptake studied	Oxygen (% air sat)	K_m (μM)	V_{max} (fmol cell^{-1} h^{-1})	Specific affinity (nl cell^{-1} h^{-1})	Yield[1] (10^{13} cells mol^{-1})	Product Specific affinity × Yield (l h^{-1} mol^{-1})
Oxygen						
glucose	80	5 63±0 11a	0 79±0 01a	0 14±0 00a	14 77±1 71a	20640
(as e donor)	0	1 38±0 02b	1 05±0 03a	0 76±0 03b	6 20±1 95a	46750
Oxygen						
glutamate	80	4 80±0 87a	0 64±0 14a	0 13±0 01a	12 25±0 29a	16370
(as e donor)	0	4 86±0 11a	1 29±0 34a	0 26±0 06a	6 57±2 07a	16060

[1] Cells produced per mol of oxygen were calculated assuming that the difference between total organic carbon present in the culture and the amount of carbon supplied was respired The respired amount of carbon was stochiometrically converted to the amount of oxygen consumed (×6 and ×4 5 mol O_2 per mol of glucose or glutamate, respectively)

Table 2 Kinetics of glucose and glutamate oxidation by *Pseudomonas chlororaphis*, grown in continuous culture (D=0 015h^{-1}, 25°C, pH 7 5) at 80% or 0% air saturation Yields and product of specific affinity and yield are also presented Values are the means (±1SE) of two replicate chemostats Significant differences between means of the different parameters for glucose and glutamate, respectively, are indicated by different letters (Tukey's test, $p<0$ 05, n=2)

Substrate uptake studied	Oxygen (% air sat)	K_m (μM)	V_{max} (fmol cell^{-1} h^{-1})	Specific affinity (pl cell^{-1} h^{-1})	Yield[1] (10^{13} cells mol^{-1})	Product Specific affinity × Yield (l h^{-1} mol^{-1})
Glucose	80	17 6±0[2]	0 14±0 00c	7 91±0 08ab[3]	56 55±3 85a	4476
	0	11 3±0 4a	0 16±0 00ab	14 00±0 20a	23 85±6 15b	3351
Glutamate	80	30 5±0 2b	0 13±0 02c	4 27±0 57b	36 60±0 80ab	1565
	0	15 1±2 1a	0 23±0 02a	15 90±3 70a	19 85±5 65b	2947

[1] Calculated as the number of cells produced per mol of glucose/glutamate consumed by the culture The amount of substrate consumed is calculated as moles of glucose/glutamate in the influent - moles glucose/glutamate in the culture at steady state

[2] Not included in statistical procedure because it is not possible to determine homogeneity of variances with 0 SE

[3] Homogeneity of variances achieved after ln transformation

Chapter 3

Effects of photoperiod on growth of and denitrification by *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima*, studied in a gnotobiotic microcosm.

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Effects of photoperiod on growth of and denitrification by *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima*, studied in a gnotobiotic microcosm.

Abstract

The emergent macrophyte *Glyceria maxima* was subjected to different photoperiods and grown with ammonium or nitrate as nitrogen source in presterilised microcosms with spatially separated root and non-root compartments The microcosms were inoculated with the denitrifying bacterium *Pseudomonas chlororaphis* The effect of the plant and the photoperiod on growth of and denitrification by *P chlororaphis* was assessed The plant had a strong positive effect on the growth of the bacteria The bacterial numbers in the root compartment of the planted microcosms were 19-32 times higher than found in the non-root sediment of the unplanted systems Lengthening the photoperiod resulted in elevated bacterial numbers due to the higher carbon exudation of the plant This effect was greater still with the nitrate-fed plants, where additional *P chlororaphis* growth could proceed via denitrification, indicating oxygen-limiting conditions in the microcosms Higher porewater N_2O concentrations in the root compartments as compared to the non-root compartments, which were highest for the long photoperiod, were also indicative of a plant-induced stimulation of denitrification. An effect of a diurnal oxygen release pattern of *G maxima* on denitrification could not be detected The gnotobiotic microcosm used in this study represents a potential system for the study of the behaviour and interactions of important bacterial groups, such as nitrifying and denitrifying bacteria where plant roots drive bacterial activity

Introduction

The presence of aerenchymatous plants can have a major impact on the composition and functioning of bacterial communities in flooded soils and sediments. The aerenchyma, which is one of the adaptations of a broad range of plant species rooting in anoxic environments (Justin and Armstrong, 1987; Blom and Voesenek, 1996), facilitates the diffusion of oxygen from the atmosphere to the roots Root-released oxygen may stimulate nitrogen cycling in the rhizosphere. The oxidation of ammonia into nitrite and nitrate by nitrifying bacteria, facilitated by the released oxygen, can subsequently stimulate denitrifying bacteria which reduce nitrate to N_2 or N_2O (Christensen and Sϕrensen, 1986; Reddy *et al.*, 1989b; Bodelier *et al.*, 1996).

Studying the impact of oxygen-releasing plants on denitrifying bacteria, and their interactions with other nitrogen converting bacteria, is extremely difficult under *in situ* conditions. Information concerning regulating factors such as the quantity of oxygen and carbon released is far from complete, as is our knowledge regarding the soil volume that is influenced by the roots under natural conditions. Furthermore reliable *in situ* activity measurements are not available and population

dynamics studies are hampered by gross underestimations as a large proportion of soil and sediment bacteria resist laboratory culturing techniques (Ward *et al.*, 1990). Moreover, the complexity of microbial activities and chemical processes in natural heterogenous soil or sediment makes assessment of *in situ* microbial characteristics extremely difficult.

One of the purposes of this study was to focus upon specific microbial interactions in an attempt to gain an insight into the complex *in situ* situation. Therefore we have developed a closed gnotobiotic soil-plant system as a model approach for plant-microbe studies. With the aid of this system, we investigated the impact of oxygen-releasing plants on denitrification in physically separated root ("macro"-rhizosphere) and non-root sediment compartments. To obtain information on the potential behaviour of denitrifying bacteria in situations where oxygen-releasing plants regulate substrate availability, we chose to manipulate the photoperiod of the plant, which undergoes daily and seasonal fluctuations. By varying the hours of daylight of the plant, which is positively related to the carbon exudation (Whipps, 1992), we aimed to vary electron donor availability both with and without excess nitrate.

We determined the influence of the emergent macrophyte *Glyceria maxima* (Hartm.) Holmb. on the growth of the heterotrophic bacterium *Pseudomonas chlororaphis*. As the bacterial strain we used is unable to reduce nitrous oxide to dinitrogen, denitrifying activity could be determined by measuring accumulated nitrous oxide in a normal dinitrogen-rich atmosphere. Plants were subjected to a 14 or 20 hour photoperiod, and effects of carbon exudation and the diurnal oxygen release pattern of the plant on denitrification were studied by monitoring the nitrous oxide concentration in the pore water of the root- and non-root compartments.

Materials and Methods

Description and operation of the model system

A schematic, vertical transection of the gnotobiotic model system, consisting of a stainless steel cylindrical sediment compartment covered by a glass cylinder, is shown in Figure 1. The sediment compartment and glass cover were connected by means of a clamping device which could be vertically positioned by a nut located in the supports of the system. In the centre of the sediment compartment a perforated stainless steel cylinder (∅ of holes: 1mm), covered on the inside with nylon gauze (mesh size 30 μm, Merrem and La Porte, Zaltbommel, The Netherlands), served to separate the rooted sediment from the bulk sediment. To sample the pore water, rhizon soil solution samplers (Eijkelkamp, Giesbeek, The Netherlands) were vertically installed to a depth of 2cm below the sediment surface. The samplers consisted of a hydrophillic porous polymer with a typical pore diameter of 0.1 μm. They were mounted on an injection needle which was connected to a second needle

via silicone tubing and a luer-lock connection. By placing an evacuated blood-collecting tube (3 ml, Terumo Venoject, Leuven, Belgium) on the second needle, pore water could be withdrawn from the system. Three pore water samplers were evenly distributed in the non-root compartment at 2.0 cm from the root compartment and 0.5 cm from the outer wall of the system. One sampling device was located in the centre of the root compartment and was also used for nutrient addition, the nutrient solution being led through a hydrophillic filter (0.2 μm, Schleicher and Schuell, Dassel, Germany). The floodwater on top of the sediment (4 cm) was replenished by a silicone tube from one of the top connections, which was led through a hydrophillic filter. The gas atmosphere in the glass cylinder was refreshed by means of a membrane pump (Whisper 500, Whisper & Silaflex, USA). The air inlet and outlet were equipped with hydrophobic PTFE inline membrane filters (0.2 μm, Gelman Science, Ann Harbour, USA). The complete system was filled with sand before being autoclaved (121°C). All materials used were selected and tested for resistance to several autoclaving cycles.

Organisms used and pre-cultivation

Plant

Glyceria maxima (Hartm.) Holmb., a common emergent macrophyte in the Netherlands, was used as a model plant as it is known to promote bacterial nitrification and denitrification in the soil surrounding its roots (Bodelier *et al.*, 1996; Both *et al.*, 1992a). *G. maxima* is highly aerenchymatous (Smirnoff and Crawford, 1983) and the inability of its roots to survive anoxia necessitates continuous oxygenation of the root tissue resulting in oxygen leakage into the rhizosphere (Brandle and Crawford, 1987; Rees *et al.*, 1987).
Seeds were collected from a *G.maxima* stand in a ditch near the institute during the 1992 growing season. They were surface sterilised by washing them for 20 minutes in 5% (v/v) $NaHClO_3$ with one subsequent rinse with 70% (v/v) ethanol and three washes with sterile demineralised water. The sterilised seeds were transferred to potato dextrose agar (Oxoid Unipath LTD, Basingstoke, UK) plates and incubated for 3 days at 28°C to check for the absence of fungi. After 3 days, non-contaminated seeds were placed on water agar (1.5% w/v) with 10% (0.3 g/l) nutrient broth (Difco, Detroit, USA) and incubated in a cabinet for germination (day/night 10h 25°C/14h 15°C, photosynthetic photon flux density 8-11 $\mu E\ m^{-2}\ s^{-1}$. Seeds which still showed signs of infection were cut out of the agar and removed. After 8 days 65% of the seeds had germinated. 17 days old seedlings were used for the experiment. All manipulations with seeds and seedlings were performed in sterile flow cabinets.

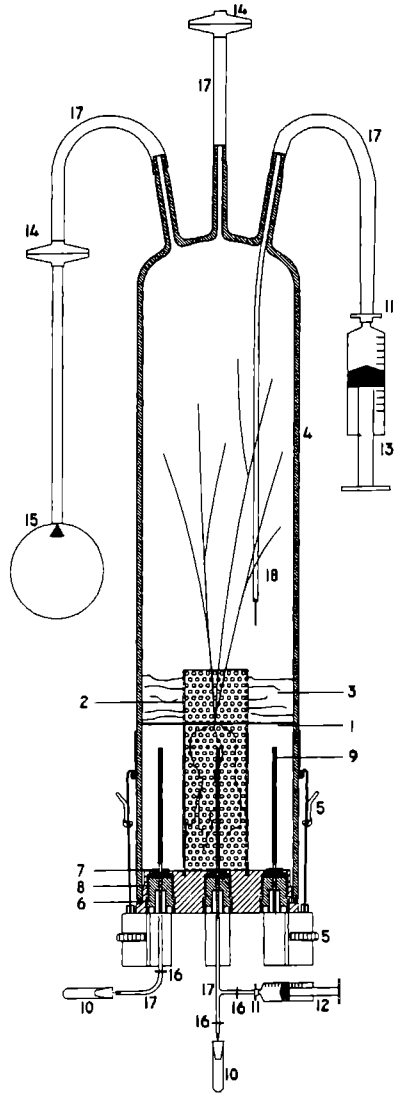

Figure 1 Graphical representation of a vertical transection of the gnotobiotic microcosm 1 stainless steel cylindrical sediment compartment (12×9cm; height×∅), 2: stainless steel root cylinder (12×4cm, h×∅), 3 floodwater layer (4cm), 4 glass cover (45×9.3cm, h×∅), 5 clamp device for pressing glass cover on soil compartment, 6. silicon greased airtight butylrubber gasket, 7 butylrubber septum, 8. bold housing harbouring the needle connecting the soil solution sampler, 9 rhizon soil solution sampler, 10 venoject sample collection tube, 11· hydrophillic filter (0 2μm), 12 disposable syringe for nutrient addition, 13 syringe for floodwater addition, 14 hydrophobic PTFE in line membrane filter (0 2μm), 15 air circulation pump, 16 tube clamps, 17 silicone tubing, 18 tube for floodwater addition

Bacterium

Pseudomonas chlororaphis ATCC 43928 is a relatively common soil heterotroph which is capable of denitrification and is able to grow in a defined medium This species is present in the root zone of *G maxima* and even tends to dominate it after amendment with nitrate (Nijburg *et al* , 1997) We used a strain only capable of reducing nitrate to nitrous oxide (Christensen and Tiedje, 1988) which is of methodological convenience The culture had been stored at -80°C Prior to inoculation of the model system, *P chlororaphis* was grown in nutrient broth batch cultures in 250 ml flasks at 25°C with 100 rpm shaking After subculturing three times, the cultures were centrifuged (10000×g, 15 minutes, 15°C) and the pellet was resuspended in phosphate buffer saline (PBS) solution (containing in grams per litre NaCl, 8 5, NaH_2PO_4 H_2O, 0 35, Na_2HPO_4 $2H_2O$, 1 34, pH 7 5) and centrifuged again This washing procedure was repeated three times The cell numbers of the washed suspension were determined microscopically using a Burker-Turk counting chamber Suspensions were diluted with PBS buffer to the desired inoculation density (see below)

Experimental design

24 model microcosms were each filled with 800 grams of dry calcareous river sand from the floodplain of the river Rhine (Bemmel, the Netherlands) Sediment characteristics were pH(H_2O) 8 3, 0 66% organic matter, and a water holding capacity of 24 51% After flooding the sand with demineralised water, the systems were closed and autoclaved twice for 60 minutes (121°C) In 16 of the systems 8 axenic *G maxima* seedlings were planted in the rhizosphere compartment The remaining 8 systems were used as unplanted controls All manipulations with the systems were performed in a sterile flow cabinet The systems were transferred to a Heraeus-Votsch HPS-1500 growth chamber (day/night cycle 16h, 22°C/8h, 15°C, relative humidity 65%, photosynthetic photon flux density at plant level ± 300 $\mu E\ m^{-2} s^{-1}$) The air flow through the cylinders was 1 0 litre min^{-1} The systems were positioned randomly in the cabinet and were rearranged regularly After 14 days, the number of seedlings per system was reduced to 4 The randomly removed seedlings, roots included, were used to estimate the initial plant dry weights Directly following thinning, 30 ml of a washed suspension of *P chlororaphis* (see above), containing $2\ 7*10^6$ cells ml^{-1}, was inoculated per system to reach a final density of $1*10^5$ cells g^{-1} dry sediment Using a syringe with a 15 cm long needle, six 5 ml aliquots were injected randomly into the sediment, pulling up the needle while emptying Most of the pore water had been removed before inoculation by means of the rhizon soil solution samplers 10 glass beakers, with the same size and filled with the same amount of sediment as the microcosms, were inoculated with *P chlororaphis* as described above These beakers, receiving

exactly the same autoclaving treatment as the microcosms, were used to estimate the initial density of *P chlororaphis* and the possible growth on residual carbon sources in the sand *P chlororaphis* numbers in these 10 beakers were determined after 3 days of incubation at 20°C by plate counting, as described below The moment of inoculation of the bacteria was regarded as the start of the experiment Plant nutrients were added weekly by injection into the root compartment via the microporous sampling device in an exponentially increasing amount The highest dose of the major elements (N, 3 mmol, P, 0 375 mmol, K, 1 125 mmol) was applied in weeks 7, 8 and 9 For the first 4 weeks all plants received NH_4^+ as the sole nitrogen source From week 5 half of the planted and unplanted systems received NO_3 as the only nitrogen source to provide *P chlororaphis* with sufficient electron acceptor and to study its denitrifying behaviour inside and outside the root zone of *G maxima* The remaining plants were kept on the NH_4^+ regime Also beginning in week 5, half of the planted systems were transferred to a growth cabinet with a photoperiod of 14 hours, and the other half was subjected to a 20 hour photoperiod The day/night temperature was set at a constant value of 20°C to prevent bacterial growth differences due to temperature differences The selection of systems for the various treatments was at random with 4 replicate microcosms per treatment

Monitoring of the pore water

In the weeks 1, 3 and 5-9, pore water samples from the root- and non-root compartments were collected prior to nutrient addition To minimize bias of the dead volume in the sampler ($\pm$0 5 ml), we discarded the first 3 ml sample The samples were immediately transferred to 4°C and were analysed for NH_4^+, NO_3, NO_2 and pH the same day

Effects of G. maxima and photoperiod on denitrification

To investigate the effects of the plant, the photoperiod and the diurnal oxygen release pattern on denitrification, porewater samples were collected from the planted and unplanted pots receiving only NO_3 This was done 2 days after NO_3 addition in week 5 and 6 at the end of the dark and light periods Accumulation of nitrous oxide in the head space was monitored at the same time by withdrawing samples after the cylinders had been devoid of air flow for 4 hours The gas-samples were analysed for the presence of nitrous oxide the same day and for NO_3 and NO_2 the next day The process of denitrification during one week was studied by sampling the pore water in week 7 daily from the moment of NO_3 addition on

Harvesting and processing of plants and sediment

After 9 weeks the systems were harvested 2 duplicate microcosms of every treatment were processed per day The floodwater was removed and stored at 4°C for further analysis Plant dry matter was determined by weighing after drying at 70°C for 2 days Moisture percentage of the thoroughly mixed sediment, was determined gravimetrically after drying at 105°C for 24 hours Mineral nitrogen content was determined in 1*M* KCl extracts (1 2 5, w/v) The numbers of *P chlororaphis* were determined by plate counts Two grams of sediment were transferred to 20 ml of PBS buffer in a 30 ml serum bottle (in triplicate) and placed on a rotary shaker for 4 hours (150 rpm, 20°C) A 1 0 ml sample was then serially diluted (tenfold serial dilutions) in PBS buffer solution Out of every dilution series, 3 dilutions were subsampled (0 1 ml) and streaked on nutrient broth agar Petri dishes (in duplicate) The Petri dishes were incubated at 28°C and the colony-forming units (CFU's) were counted after 1 week of incubation For calculating the number of CFU's per gram of dry sediment the dilution was used which showed 50-100 CFUs per plate

Chemical analyses

NH_4^+, NO_3 and NO_2 in pore water samples, sediment extracts, nutrient solutions and floodwater were analysed using a Technicon Traacs 800 autoanalyzer (Technicon Instr Corp , Tarrytown, USA) Nitrous oxide in porewater samples was measured by injecting head space samples from venoject tubes into a gas chromatograph (Carlo Erba GC 6000) equipped with a Hayesep Q column (80°C), an electron capture detector (ECD) (nitrous oxide < 100 ppmv) and a hot wire detector (HWD)(nitrous oxide > 100 ppmv) The total amount of nitrous oxide in the gas and liquid phases of the venoject was calculated using the Bunsen absorption coefficient for nitrous oxide (0 632, 20°C, 1 atm, Tiedje, 1994) The chemical composition of the plant material was analysed using dried, ground (0 5 mm) plant material as described by Troelstra (1983) and Troelstra *et al* (1995) The proportion of carboxylate and organic nitrogen were determined to provide an indication of the relative ammonium/nitrate uptake by plants (Troelstra 1983)

Statistical analyses and calculations

All statistical analyses were performed using the SATISTIX analytical software package (NH Analytical Software, St Paul MN 55117, USA) Data were checked for normality by means of the Wilk-Shapiro test Treatment effects of nitrogen form and photoperiod on plant biomass, plant chemical composition and numbers of *P chlororaphis* were analyzed by a two way ANOVA Comparisons of means were performed by Tukey's test Pairwise comparisons of pore water NO_3 , NO_2

and nitrous oxide concentrations at the end of light and dark period were analysed using the two-sample t-test which accounts for inequality of variances as described by Snedecor and Cochran (1982)
Porewater nitrate, nitrite and nitrous oxide concentrations during week 7 were analysed per day of sampling by Tukey's test Data were either ln or square root transformed to obtain homogeneity of variances
Relative growth rates (RGR) per week were calculated using initial (W1) and final dry weights at harvest (W2) according to equation 1

$$\mathbf{RGR = (lnW2-lnW1)/(t2-t1)} \quad (1)$$

Results

General performance of the microcosm

The roots of the plant did not penetrate the nylon mesh and filled the root cylinder completely at harvest time Visual evidence for release of oxygen by the roots was present in the form of Fe_2O_3 traces on the nylon mesh at the spots where the roots had been in contact with the mesh All planted, nitrate-fed microcosms remained free of contamination during the complete experimental period and contained only *P chlororaphis* The remaining microcosms were contaminated with 3 different bacterial strains, with contamination reaching up to 45% of the total bacteria present In all other microcosms, *P chlororaphis* represented more than 75% of the bacteria present The contaminating bacteria were characterised as an aerobic, Gram-negative, non-motile rod which formed transparent yellow colonies, an aerobic Gram-negative motile rod which formed non-transparent yellow colonies and a Gram-positive, coccoid- shaped bacterium capable of fermentation in Tryptic Soy Broth forming grey/white colonies None of the contaminants could utilise nitrate as an electron acceptor and were also not present in the beakers used for the determination of the initial cell numbers
The dynamics of the added ammonium and nitrate and pH of the pore water could be monitored during the entire experiment Ammonium and nitrate concentrations in the unplanted systems increased approximately exponentially following nutrient addition (Fig 2 A,B) Compared to the non-root compartment, ammonium concentrations in the pore water of the root compartment were always higher, whereas nitrate concentrations were equal In the planted systems, ammonium and nitrate also began to accumulate from week 7 An increasing amount of the nitrate in the unplanted microcosms was reduced to nitrite, until week 7 after which nitrite remained constant (Fig 2C) Very little nitrite accumulation occurred in the planted systems

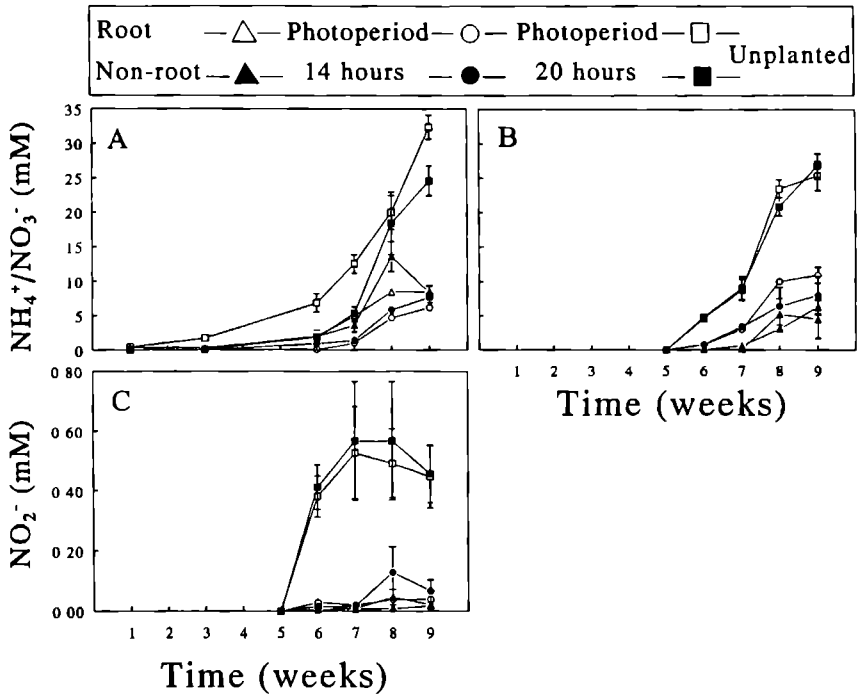

Figure 2 Porewater concentrations of NH_4^+ (A), NO_3 (B) and NO_2 (C) in gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis* and receiving only NH_4^+ (A) or NO_3 (B,C) as nitrogen source. The plants were grown under two different photoperiods (14 vs 20 hours) Untill week 5, all microcosms received ammonium, application of different nitrogen sources occurred from week 5 on Values are means (±SE) of 4 replicate microcosms (except for unplanted microcosms + NH_4^+ where n=3)

Pore water pH was in the range of 7.9-8.3 at the beginning of the experiment (data not shown). In the planted microcosms pore water pH of the root compartments decreased to ±7.0, whereas the pH in the non-root compartments and in the unplanted systems remained unchanged. Following the separation in ammonium and nitrate supply (week 5), the pH in the root compartments of the ammonium-fed plants continued to decrease to values of 6.3-6.4. The pH in the root compartment of the ammonium-fed plants was always lower than for the corresponding non-root sediment. In the planted columns with nitrate, pH in the root- and non-root compartment reached the same level of pH 7.0 one week after starting the nitrate supply. From week 7 on there was a steep increase in pH in both compartments of the planted nitrate-fed microcosms to values of 7.7 and 8.1 in root- and non-root compartment, respectively. Photoperiod did not have any major influence on pore water pH. It is also noteworthy that the pH in the central "root" cylinder of the unplanted systems was often lower than in the corresponding non-root

compartments
Recovery of the added nitrogen was in the range of 51-69% and was the same for planted as well as unplanted systems (data not shown) Nitrate supply tended to lead to lower recoveries. Nitrate-fed plants acquired less nitrogen than ammonium-fed plants, 35-37% vs 48-51% of the added nitrogen, respectively. Photoperiod seemed to have had no effect on overall nitrogen balances, except for the planted nitrate-fed microcosms where a longer photoperiod tended to result in higher N-loss.

Plant parameters

With ammonium as nitrogen source, a longer photoperiod led to higher biomass production, whereas with nitrate-fed plants biomass formation was not influenced by photoperiod (Table 1). Total dry weight of plants subjected to a 14 hour photoperiod did not differ for ammonium- or nitrate-fed plants. The ratio of shoot and root biomass was equal for all the treatments. The relative growth rate (RGR) of *G.maxima* was in the range of 0.83-0.89 week^{-1} (Table 1). The growth rate of ammonium-fed plants with the long photoperiod was significantly higher than the growth rates of the other treatments, which showed no significant difference with each other. Analysis of variance indicated that total biomass production was affected by both photoperiod and nitrogen source, RGR only by the photoperiod and shoot-root ratio by none of the treatments (Table 2).

Table 1 Total dry weight, shoot-root ratio (S/R) and relative growth rate (RGR) of *Glyceria maxima*, grown in gnotobiotic microcosms subjected to two different photoperiods (14 vs 20 hours) and with ammonium or nitrate as nitrogen source The sediment was inoculated with *Pseudomonas chlororaphis* ($1*10^5$ cells g^{-1} dry sediment) Values are means ($\pm$1SE) of 4 replicate microcosms Different letters per column indicate significant differences (Tukey's test, $p<0.05$)

Photoperiod/ N-source	Total dry weight(g)	S/R ratio	RGR (week^{-1})
14h/NH_4^+	3 77$\pm$0 52a	1 78$\pm$0 30a	0 84$\pm$0 014a
14h/NO_3	3 27$\pm$0 13a	1 14$\pm$0 02a	0 83$\pm$0 004a
20h/NH_4^+	6 13$\pm$0 51b	1 36$\pm$0 17a	0 89$\pm$0 008b
20h/NO_3	3 91$\pm$0 16a	1 25$\pm$0 07a	0 85$\pm$0 005a

Table 2 ANOVA results (degrees of freedom, F- and p-values) of total dry weight, shoot-root ratio (S/R) and relative growth rate (RGR) of *Glyceria maxima* grown in a gnotobiotic microcosm subjected to two photoperiods (14 vs 20 hours) and with ammonium or nitrate as nitrogen source

Treatment	df	Variable					
		Total dry weight		S/R ratio		RGR	
		F	P	F	P	F	P
Light (L)	1	12 98	0 0036	0 87	0 3691	13 98	0 0028
N-form (N)	1	15 78	0 0018	4 61	0 0528	4 68	0 0514
L*N	1	5 14	0 0426	2 28	0 1571	1 07	0 3224

Table 3 Chemical composition of Glyceria maxima (whole plant) grown in a waterlogged gnotobiotic microcosm subjected to two photoperiods (14 vs 20 hours) and with ammonium or nitrate as nitrogen source The sediment in the microcosms was inoculated with *P chlororaphis* ($1*10^5$ g^{-1} dry sediment) Values are means for 4 replicate microcosms Ion concentrations are expressed as meq kg^{-1} dry matter, organic nitrogen as mmol N kg^{-1} dry matter Different letters indicate significant differences (Tukey's test, $p<0\ 05$) between treatments

Variable	Photoperiod	14h		20h	
	N-form	NH_4^+	NO_3	NH_4^+	NO_3
K^+		476 a	402 ab	237 c	330 bc
Na^+		252 a	314 a	211 a	318 a
Ca^{2+}		245 a	238 a	232 a	239 a
Mg^{2+}		79 a	77 a	52 b	70 ab
NH_4^+		12 a	6 ab	7 ab	5 b
H_2PO_4		78 a	57 ab	34 b	47 b
NO_3		1 b	91 a	0 25 b	41 a
Cl		462 a	299 b	269 b	271 b
SO_4^{2-}		146 a	101 a	74 a	127 a
Carboxylate (C-A)		395 bc	510 a	356 c	476 ab
Organic N		1640 a	1158 b	892 b	932 b
C-A/N_{org}		0 25 a	0 44 bc	0 41 c	0 53 b

Lengthening of the photoperiod resulted in lower K^+, Mg^{2+}, H_2PO_4, Cl, and organic nitrogen concentrations for the ammonium-fed plants (Table 3). There were no differences in the chemical composition of the nitrate-fed plants between the two photoperiods. When comparing ammonium- and nitrate-fed plants per photoperiod, it appeared that the carboxylate concentration (cations-inorganic anions) of the nitrate-fed plants was higher for both photoperiods. The carboxylate-organic nitrogen ratio was also higher for the nitrate-fed plants compared to the plants using ammonium as a nitrogen source (Table 3). This ratio was elevated only in the case of the ammonium-fed plants by the extension of the photoperiod. The analysis of variance revealed main effects of photoperiod on K^+, Mg^{2+}, H_2PO_4, NO_3 and organic nitrogen concentrations in the plant (Table 4). Nitrogen source had main effects on Na^+, NH_4^+ NO_3, Cl and carboxylate concentration. Interactions between photoperiod and form of N were found for the K^+, H_2PO_4, Cl, SO_4^{2-}, NO_3^- and organic N concentration. The $(C\text{-}A)/N_{org}$ ratio was affected by the photoperiod via the organic nitrogen and by the nitrogen nutrition via the carboxylate concentration. There was no interaction between light regime and nitrogen source in relation to the $(C\text{-}A)/N_{org}$ ratio.

Table 4 ANOVA results (degrees of freedom, F- and p values) of the main treatment effects (light, nitrogen form), and their interactions, on the chemical composition of *Glyceria maxima*, grown in a waterlogged gnotobiotic microcosm subjected to two different photoperiods (14 vs 20 hours) and with ammonium or nitrate as nitrogen source The sediment was inoculated with *Pseudomonas chlororaphis* ($1*10^5$ g^{-1} dry sediment)

		Treatment					
		Light (L)		N-form (N)		L*N	
Variable	df	F	p	F	p	F	p
K^+	1	25 10	0 0003	0 010	0 7631	7 28	0 0194
Na^+	1	0 30	0 5968	6 39	0 0265	0 45	0 5144
Ca^{2+}	1	0 24	0 6365	0 00	0 9809	0 25	0 6246
Mg^{2+}	1	11 10	0 0060	2 27	0 1579	3 94	0 0706
NH_4^+	1	4 56	0 0540	5 94	0 0313	1 75	0 2106
H_2PO_4	1	16 60	0 0015	0 30	0 5950	6 54	0 0251
NO_3	1	5 95	0 0312	39 30	0 0000	5 48	0 0373
Cl	1	3 02	0 1080	7 84	0 0160	8 02	0 0151
SO_4^{2-}	1	1 70	0 2172	0 05	0 8243	7 47	0 0182
Carboxylate	1	2 17	0 1666	22 28	0 0005	0 01	0 9169
Organic N	1	20 08	0 0008	4 15	0 0644	5 77	0 0334
$C\text{-}A/N_{org}$	1	16 01	0 0018	27 18	0 0002	1 19	0 2966

Growth of P. chlororaphis in the microcosms

The numbers of *P. chlororaphis* in the root compartments of the planted systems were all substantially higher than in the non-root sediment (Fig. 3). CFU's were in the range of 3.8×10^{7}-1.1×10^{8} and 3.8×10^{6}-2.8×10^{7} g^{-1} dry sediment in the root- and non-root sediment, respectively. The planted/unplanted CFU ratios of the rooted sediment for ammonium- and nitrate-fed plants, respectively, were 12 and 15 for the short photoperiod and 19 and 32 for the long photoperiod. Elongation of the photoperiod resulted in significantly ($p<0.05$) higher growth of *P. chlororaphis* in the root- and non-root compartments of both ammonium- and nitrate-fed plants. From the ANOVA in Table 5 it is evident that growth of *P. chlororaphis* in the root zone of *G. maxima* was affected by the interaction of photoperiod and nitrogen source while growth in the non-root sediment was affected separately by photoperiod and nitrogen nutrition.

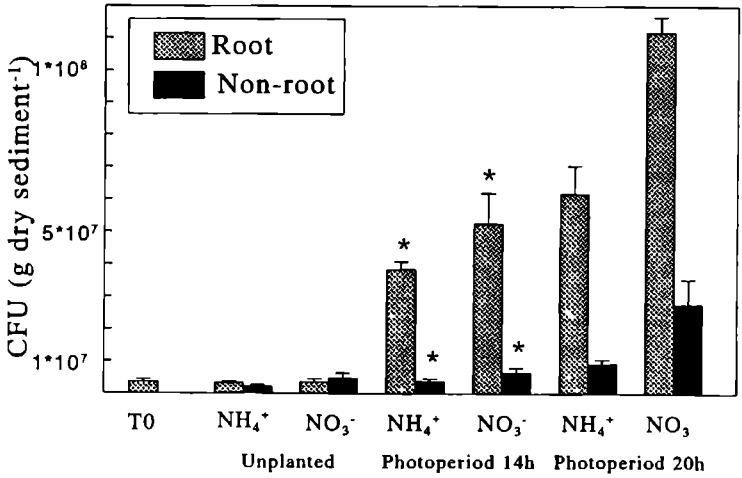

Figure 3. The number of colony-forming units of *Pseudomonas chlororaphis* in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glycerta maxima* The plants were grown under two different photoperiods (14 vs 20 hours) and with NH_4^+ or NO_3^- as nitrogen source T0 is the initial inoculum density determined 3 days after inoculation. Bars are means ($\pm$SE) of 4 replicate microcosms (except for unplanted + NH_4^+ where n=3) Asterisks indicate significant differences between the 14- and 20 hour photoperiod ($p<0$ 05, One way anova)

Table 5 ANOVA results (degrees of freedom, F- and p-values) of the number of colony- forming units of the bacterium *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima* and in the non-root sediment The plants were grown in waterlogged gnotobiotic microcosms subjected to two photoperiods (14 vs 20 hours) and with ammonium or nitrate as nitrogen source

		Variable			
		CFU root compartment		CFU non-root compartment	
Treatment	df	F	P	F	P
Light (L)	1	36 23	0 0001	11 39	0 0055
N-form (N)	1	21 13	0 0006	7.20	0 0199
L*N	1	7 05	0 0210	4 02	0 0680

Effects of G. maxima and photoperiod on denitrification by P. chlororaphis

The pore water nitrous oxide concentrations were determined for planted and unplanted microcosms in week 5 and 6 at the end of the light and dark period 2 days after nitrate addition and the results are presented in Figure 4. There were no significant differences in nitrous oxide concentrations between the end of the light and dark period. This held true for both weeks 5 and 6 of the experiment. The presence of the plant resulted in an elevated nitrous oxide concentration, compared to the unplanted systems. The plant-induced nitrous oxide accumulation occurred in particular in the pore water of the root compartments. Despite the obvious trend toward higher nitrous oxide concentrations in the root compartments of the planted systems differences often failed to reach significance due to the high variation in nitrous oxide concentrations (CV 47-184%) between the replicates. The head space of the microcosms did not contain detectable amounts of nitrous oxide at any sampling event. A substantial amount of the added nitrate was reduced to nitrite, particularly in the unplanted microcosms where nitrite concentrations amounted to 0.35-0.37 *mM* in week 6 (data not shown). Pore water nitrite concentrations in the planted systems were always lower than 0.10 *mM* and there was no photoperiod effect.

In week 7 of the experiment, porewater nitrous oxide concentrations were monitored daily (Fig. 5), with the first sample being taken 5 hours after nitrate addition on the first day of the week. The nitrous oxide levels at day 1 of week 7 were in the same range as the values at day 2 of week 6. There was a distinct plant effect on nitrous oxide production during the week. Concentrations in the root compartment of the plants subjected to the long photoperiod, increased significantly to a value of 21 μM on day 2, and subsequently decreased again to levels not significantly different from the non-root sediment and the unplanted systems In the

planted microcosms subjected to the short photoperiod, this elevation was much weaker and statistically not significant. In the unplanted systems nitrous oxide levels gradually increased averaging 4 μM on day 6. As in weeks 5 and 6, the variation in the nitrous oxide concentrations between replicate microcosms was substantial.

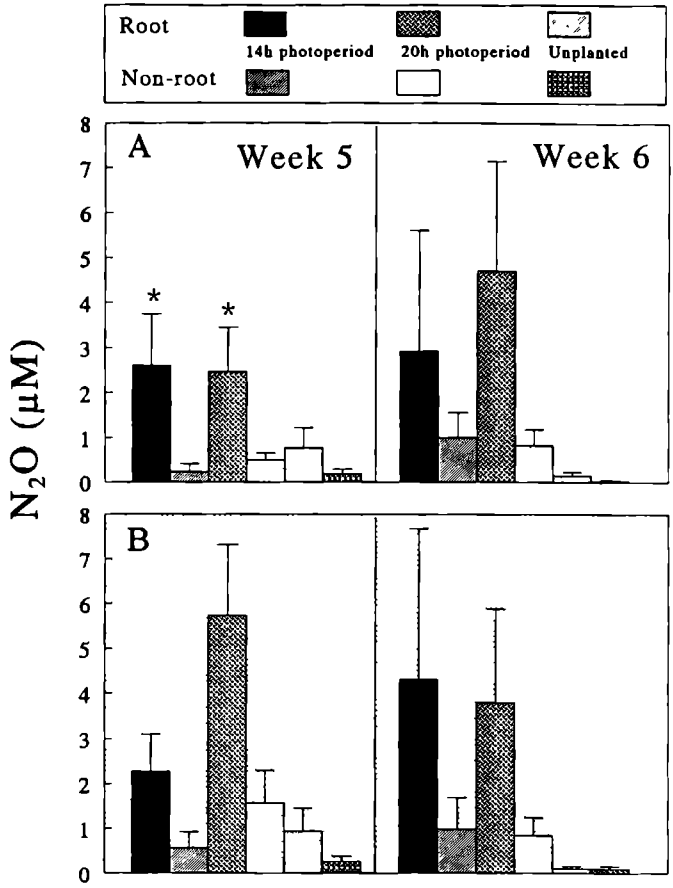

Figure 4: Porewater N_2O concentrations at the end of the light (A) and dark period (B), two days after NO_3 addition in week 5 and 6, in gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis*. The plants were grown under two different photoperiods (14 vs 20 hours) and with NO_3 as nitrogen source. Bars indicate means ($\pm$SE) of 4 replicate microcosms. Asterisks indicate significant differences between root and non-root sediment ($p < 0.05$, Rank-Sum test).

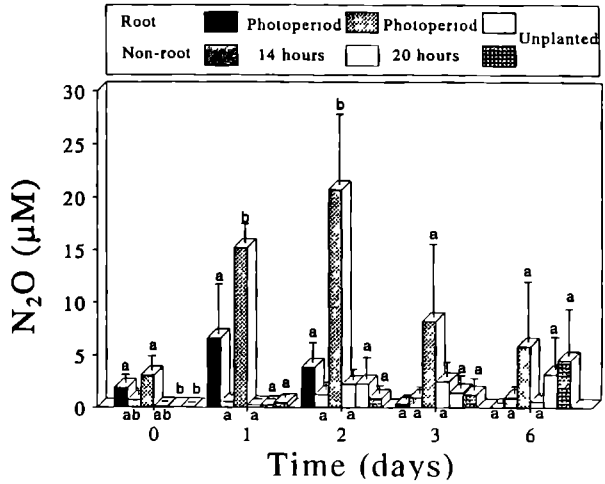

Figure 5 Porewater N_2O concentrations during week 7 in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis* The plants were grown under two different photoperiods (14 vs 20 hours) and with NO_3 as nitrogen source Bars indicate means (±SE) of 4 replicate microcosms Different letters indicate significant differences between means of one sampling day ($p<0$ 05, Tukey's test) Data were ln or square root transformed to obtain homogeneity of variances The untransformed data are shown.

Discussion

General performance of the gnotobiotic microcosms

To the best of our knowledge, this represents the first report describing the use of this type of gnotobiotic microcosm. Closed, gnotobiotic soil-plant systems have been used in plant-microbe interaction studies, but have usually consisted of simple test tube set-up use for short incubation periods with very young seedlings (Bennett and Lynch, 1981; Turner and Newman, 1984). Neither of these studies provided information concerning sterility or contamination of the system. A larger experimental set-up was used by Trofymow *et al.* (1987), who studied rhizodeposition and ammonium depletion by means of a split root system with axenic oat roots. These authors regarded their system as being sterile when less than 50 colonies per ml were found in a 10-fold dilution of a soil suspension. Both (1990) constructed a gnotobiotic system for studying spatial distribution of nitrifying bacteria around roots of *Plantago lanceolata*. This elegant system was, however, contaminated with a *Pseudomonas* species. In our experimental design we succeeded in keeping a number of the microcosms free from any contamination during the full 3-month experimental period, showing that the system can

potentially be used for gnotobiotic studies The systematic contamination of a number of the microcosms indicated that addition of the nutrient solutions was a common source of infection Obviously, the 0 2μm hydrophillic filter and the 0 1μm porous polymer material of the sampling device were not always 100% effective in the exclusion of contaminants

By regarding the completely rooted, central root cylinder, as a "macro-rhizosphere" from which carbon and oxygen are released into the surrounding non-root compartment, we could study microbial dynamics in the sediment layers adjacent to the root cylinder by subdividing the non-root sediment at the end of the experimental period This principle has already been used in several studies in different context (Boero and Thien, 1979, Helal and Sauerbeck, 1983, Klemedtsson *et al* , 1987a) Klemedtsson *et al* (1987a) were the only authors looking at nitrification and denitrification in a non-gnotobiotic system The "macro-rhizosphere" principle overcomes the difficulties of defining the rhizosphere and facilitates the study of microbial processes at defined distances from the roots Other authors used an artificial root to overcome the traditional problems (Martens, 1982, Odham, 1986) The problem of artificial root systems is that the amount and dynamics of carbon and oxygen release is set by the investigators, and it is questionable whether this reflects the "natural" dimensions of such highly variable processes A close approximation of the natural situation was achieved by Hϕjberg and Sϕrensen (1993), who created a gel-stabilised system, in which microgradients of bacterial activity could be measured near single roots The use of a gel matrix in combination with an intact root system is closer to the natural situation in comparison to artificial root systems The mechanical resistance created by the gel matrix which can influence oxygen (Engelaar *et al* , 1993) and carbon (Barber and Gunn, 1974) release, will, however, differ

The nitrogen added to our systems could not be completely recovered In the ammonium-fed microcosms, 31-37% of the supplied nitrogen was not recovered This can partially be explained by the method of nutrient supply, which occurred in small concentrated amounts Apparently, a part of the nitrogen is retained in the dead volume of the hydrophillic filter + tubing of the rhizon soil solution sampling device Although the sampling device was always rinsed with demineralised water after nutrient addition, we calculated that an amount of 12% of added nitrogen may not have reached the root compartment Furthermore, ammonia volatilization may very well have occurred at the slightly alkaline pore water pH in the unplanted systems

Growth of G. maxima

Our model plant, *G maxima*, grew normally in the closed cylinders The relative growth rate (0 83-0 89 week ^{1}) was slightly lower than seedling RGR in natural situations (1 0-1 4 week ^{1}, Grime *et al* , 1988) This is probably due to the light-

limited conditions created by the low photosynthetic photon flux density of ± 300 $\mu E\ m^{-2}s^{-1}$ ($\approx 65\ W\ m^{-2}$), which is probably below the photosynthetic saturation. The chemical composition of the plants indicated that nutrient supply was sufficient. The plant content of the major elements (N; 1.70%, P; 017%, K; 1.41%, Ca; 0.24%, Mg; 0.08% of the dry matter) agreed very well with values for natural *G. maxima* stands at eutrophic freshwater locations (Dykyjova, 1978). The carboxylate-organic nitrogen ratio may give an indication of the relative amount of nitrate, as opposed to ammonium, taken up by the plant (Troelstra, 1983). In the case of ammonium uptake this ratio will be lower as compared to nitrate uptake due to the lower carboxylate formation. The values in this study agree with this concept and may in principal be used as reference values for studies where the amount of nitrate acquired by the plant is determined by nitrification and denitrification. The differences between the ammonium- and nitrate-fed plants was not very large which is common in the *Gramineae* (van Egmond, 1978). It is questionable, however, whether such a subtle difference will be reflected by the $C\text{-}A/N_{org}$ when the nitrate production is relatively small and denitrification high. Furthermore, the elevated $C\text{-}A/N_{org}$ ratio in the ammonium-fed plants was related to the elongated photoperiod and not to a relative increase in nitrate nutrition, which may be problematic for future experimental interpretations. The photoperiod changed the organic N content of the ammonium-fed plants drastically, but did not affect the carboxylate content, which was influenced by the form of nitrogen as expected. In our experimental setting it might be more useful to use the carboxylate content as indicator of the relative nitrate uptake instead of the $C\text{-}A/N_{org}$ ratio's. The values in the present study can be used as reference values in future experiments.

Effect of G. maxima and photoperiod on growth of and denitrification by P. chlororaphis.

The rhizosphere effect on the growth of *P. chlororaphis* found in this study (R/S ratios 12-32, i.e. planted/unplanted) is in the range found in fertile soils planted with arable crops (Woldendorp, 1981). It was obvious that the plant also stimulated the bacterial population in the non-root sediment. The effect of the plant on the non-root sediment was probably greatest directly adjacent to the root compartment, and subdividing the non-root sediment in layers of increasing distance from the "macro-rhizosphere" should allow for the study of microbial population dynamics across a gradient of root-released substrates.
Although we were interested in the impact of plant-carbon or oxygen release, as affected by photoperiod, on the growth of and denitrification by *P. chlororaphis* it is only possible to interpret the amount of oxygen and carbon released by *G. maxima* qualitatively. The total number of *P. chlororaphis* cells formed per microcosm due to the presence of *G. maxima* averaged 5.52×10^9 and 6.35×10^9

cells for the ammonium- and nitrate-fed systems, respectively, of the 14 hour photoperiod experiment. For the 20 hour photoperiod experiment these values were 1.25×10^{10} and 2.94×10^{10}. In the nitrate-fed systems there is an excess of electron acceptor which means that elevation of the photoperiod led to an increase in the plant-derived carbon, which could be converted into bacterial biomass, with a factor of 4.6. This value was only 2.3 in the ammonium-fed systems. The stimulation of carbon exudation and higher bacterial numbers associated with the plants due to a longer photoperiod was also found by Whipps (1984). Apparently, there was a relative shortage of plant-derived oxygen when taking the higher cell numbers into account when nitrate was available. The total cell numbers in the nitrate-fed microcosms were 1.2 and 2.4 times the numbers found for the ammonium-fed systems for the 14- and 20 hours photoperiod, respectively. Hence, the relative shortage of oxygen compared to the available amount of carbon increases with the hours of daylight. Extrapolation to the field situation predicts better growth conditions for facultatively aerobic rhizosphere bacteria in summer and autumn and less favourable conditions in winter and spring. The ability to regulate the degree of oxygen deprivation allows one to mimic the field situation more closely providing the prospect of studying the competitive abilities of nitrifying bacteria as they might occur in nature.

The plant-stimulated denitrification in the microcosms is a phenomenon which has been described by many studies (e.g Woldendorp, 1963; Stefanson, 1972; Prade and Trolldenier, 1990). The impact of the plant on denitrification in relation to its photosynthesis was described by Scaglia *et al* (1985) and Bakken (1988). Since nitrate is not limiting, the effect of the photoperiod on denitrification in our study must be due to the higher carbon availability. However, the nitrous oxide production pattern in week 7 revealed an initial increase in denitrification followed by a decrease which is consistent with the decreasing nitrate availability. The bacteria are probably outcompeted by the plant for nitrate, but it is also possible that nitrous oxide escapes from the sediment via the aerenchymatous tissue of the plant as was demonstrated by Mosier *et al.* (1990) and Reddy *et al.* (1989). However, we were unable to detect any nitrous oxide in the head space of the microcosms.

Providing *P. chlororaphis* with nitrate in week 5 led to immediate nitrite accumulation, which is an indication of excess electron acceptor, leading to an incomplete denitrification reaction (R.A. Kester, personal communication). This appears even more likely if one considers that nitrite accumulation in the unplanted systems, which have a lower electron donor availability, is substantially higher. Effects of the diurnal photosynthetic cycle on denitrification could not be detected. The oxygen released by the plants during the day might very well inhibit denitrification, as was found by Christensen and Sϕrensen (1986) in the root zone of the macrophyte *Litorella uniflora (L.) Aschers*. The high variation in nitrous oxide pore water concentrations between the replicate microcosms might also

hamper detection of possible differences between day and night measurements The system we describe in this study provide a potential means of studying complex microbial interactions in the vicinity of roots The "macro" rhizosphere principal, can be used as a simplification of the highly complicated natural rhizosphere, enabling the study of potential interactions of selected members of microbial communities Although this remains a model approach, the dimensions and gradients of the plant-derived microbial substrates (i e carbon, oxygen) are still governed by the plant thus allowing for a closer approximation of the natural situation The system does claim to match *in situ* processes but can help to describe the specific behaviours and interactions of important functional bacterial groups, whose activities are dictated by the plant

Chapter 4

Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima.*

With H. Duyts, C.W.P.M. Blom and H.J. Laanbroek

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Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima*

Abstract

The population dynamics of the chemolitho-autotrophic nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were studied in gnotobiotic microcosms fed with ammonium in response to the presence or absence of the emergent macrophyte *Glyceria maxima* and the heterotrophic denitrifying bacterium *Pseudomonas chlororaphis* By subjecting the plants to different day lengths, the effect of possibly limiting factors (i e oxygen and ammonium) on the interactions between the nitrifiers and the denitrifying bacterium could be analysed The presence of the plant had no effect on the growth of nitrifiers suggesting that, in addition to radial oxygen loss from the roots, also other sources of oxygen (e g diffusion from the water layer) were important for nitrification. Potential nitrifying activities were suppressed by *G maxima* due to ammonium uptake by the plants Elongation of the day length in combination with the presence of *G maxima* led to an increase in the number of *P chlororaphis* The presence of *P chlororaphis* suppressed potential nitrifying activities and the growth of *N winogradskyi*, but the growth of *N europaea* was not affected Potential denitrifying activities were stimulated by the plant, but showed no correlations with nitrifier activities or numbers Apparently ammonium, and not oxygen, was the limiting factor for nitrification in the root zone of *G maxima* However, when the plant did not deplete the ammonium pool, *P chlororaphis* could repress the nitrifiers indicating the latter's poor competitive status with respect to oxygen when the presence of root exudates allows for heterotrophic oxygen consumption

Introduction

At anoxic/oxic interfaces in soils and sediments, ammonia can be oxidised to nitrate and subsequently reduced to N_2 or N_2O, leading to nitrogen loss from the ecosystem and emission of the greenhouse gas nitrous oxide (Firestone and Davidson, 1989). In sediments, the upper few millimeters and the rhizosphere of oxygen-releasing, aerenchymatous plants, are potential interfaces for coupled nitrification-denitrification As denitrification depends upon the presence of NO_3 nitrogen loss due to denitrification is dependent upon the activity of the aerobic chemolithotrophic nitrifying bacteria. Ammonia-oxidising bacteria convert ammonia into nitrite which is oxidised to nitrate by nitrite-oxidising bacteria. Oxygen and ammonia availability are critical regulating factors of the nitrification process. All oxygen-consuming organisms and processes will compete for available oxygen in sediments, whereas ammonia availability will be determined mainly by plant uptake and bacterial immobilisation and consumption.
Nitrifying bacteria are assumed to be poor competitors for limiting amounts of oxygen (Sharma and Ahlert, 1977; Prosser, 1989; van Niel, 1991) and have been shown to lose the competition with plants (Engelaar *et al.*, 1991; Verhagen *et al*,

1994) and bacteria (Verhagen and Laanbroek, 1991) for limiting amounts of ammonia Nevertheless, growth and activity of these organisms in the root zone of oxygen-releasing plants has been demonstrated to occur by means of cell counts, activity measurements and stimulated denitrification (Christensen and Tiedje, 1988, Reddy *et al* 1989b, Engelaar *et al*, 1995, Bodelier *et al* , 1996) The survival mechanisms of aerobic nitrifiers in this environment, however, remain obscure Longevity, especially under oxygen-poor conditions seem to be a distinct trait of some nitrifiers (Cavari, 1977, Hall and Jeffries, 1984, Jensen *et al* , 1993) Comparing nitrification in a freshwater lake sediment and in a permanently oxic dune soil, Bodelier *et al* (1996) found that ammonia oxidisers from sediment had higher affinities for oxygen than nitrifiers from well aerated terrestrial soils Moreover, following a period of anoxia, these bacteria became fully active immediately after return to oxic conditions, whereas ammonia oxidisers from the dune soil exhibited a lag time prior to the onset of nitrifying activity Competitive success of nitrifiers for limiting amounts of oxygen with heterotrophic organisms has never been reported Based on pure culture studies (Bodelier and Laanbroek, 1997) and field data (Bodelier *et al* , 1996) it can be concluded that ammonia-oxidising cells will not lose the competition from heterotrophic cells on the basis of their oxygen uptake kinetics Yield characteristics and uptake kinetics of the electron donor are probably more important factors for the functioning of ammonia oxidisers in low oxygen habitats Thus, in the rhizosphere of oxygen-releasing plants, the availability of substrates (i e organic substrates and ammonia) for nitrifying and heterotrophic bacteria will also influence the interactions of these organisms under oxygen-limiting conditions Thus, in addition to releasing oxygen, the plant will also influence the interactions between nitrifying and denitrifying bacteria by releasing organic carbon and taking up ammonia

The aim of this study was to test whether, and to what extent, nitrifiers are able to survive in the rhizosphere of oxygen-releasing plants and to investigate the possible influence of interactions with a denitrifying bacterium Due to the elevated carbon release and subsequent stimulation of heterotrophs by *Glyceria maxima* (Bodelier *et al* , 1997), day length could be used as a "natural" variable to study plant-induced effects on bacterial interactions We studied the population dynamics of the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in the presence or absence of the denitrifier *Pseudomonas chlororaphis*, in gnotobiotic microcosms planted with *Glyceria maxima* In this system, many of the uncertainties of studies with natural soils and sediments, such as underestimations of bacterial numbers due to limitations of culture methods, heterogeneity of soil or sediment, and definition of the rhizosphere can be circumvented The photoperiod imposed on the plant and the distance to the roots were used as variables to obtain information on the interactions between nitrifiers and denitrifiers in the plant-mediated oxic/anoxic zones

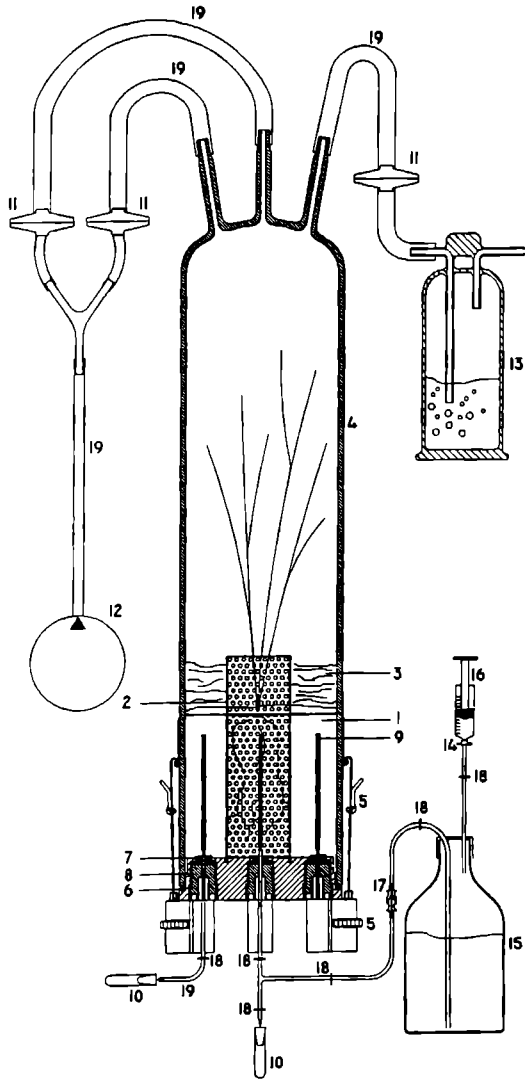

Figure 1 A vertical cross-section of the gnotobiotic microcosm 1 stainless steel cylindrical sediment compartment (12×9cm, height×∅), 2 stainless steel root cylinder (12×4cm, h×∅), 3 floodwater layer (4cm), 4 glass cover (45×9 3cm, h×∅), 5 clamp device for pressing glass cover on soil compartment, 6 silicon greased airtight butylrubber gasket, 7 butylrubber septum, 8 bold housing harbouring the needle connecting the soil solution sampler, 9 rhizon soil solution sampler, 10 venoject sample collection tube, 11 hydrophobic PTFE in line membrane filter (0 2μm), 12 air circulation pump, 13 NH_3 trap filled with H_3BO_3, 14 hydrophilic filter, 15 500 ml flask for nutrient addition, 16 disposable syringe for pressurising nutrient solution, 17 glass tube connection, 18 tube clamps, 19 silicone tubing

Materials and Methods

Description and operation of the model system

A schematic, vertical transection of the gnotobiotic model system used is shown in Figure 1 This system represents a modified version of that described by Bodelier *et al* (1997) The most important modification was the way in which nutrients and floodwater were added Nutrient solution and floodwater was autoclaved in a flask which was connected to the rhizon soil solution sampling device in the root compartment The oxygen-free nutrient solution was transferred from the flask to the microcosm by pressurising the flasks by means of a syringe filled with N_2 gas Before addition of nutrients, the air present in the silicone tubing was withdrawn by means of a vacuum sample-collection tube (Terumo, Belgium) Another modification was the NH_3 trap connected to the air outlet of the system which contained boric acid (H_3BO_3, 100*mM*, pH<4 8)

Organisms used and pre-cultivation

Plant

Glyceria maxima (Hartm) Holmb , a common emergent macrophyte in the Netherlands, was used as a model plant as it is known to produce stimulating effects on nitrification and denitrification (Both *et al* , 1992a, Bodelier *et al* , 1996) *G maxima* is highly aerenchymatous (Smirnoff and Crawford, 1983) and the inability of its roots to survive anoxia necessitates the continuous oxygenation of the root tissue which leads to oxygen leakage into the rhizosphere (Brandle and Crawford, 1987, Rees *et al* , 1987) Sterile seedlings were obtained by surface sterilisation of the seeds with $NaHClO_3$ as described by Bodelier *et al* (1997)

Bacteria

Nitrosomonas europaea ATCC 19718 and *Nitrobacter winogradskyi* ATCC 25391 were used as the nitrifying bacteria The bacteria were cultivated separately in batch cultures in 500 ml flasks containing 200 ml medium of the following composition (mg/l) $(NH_4)_2SO_4$, 660 (*N europaea*) or $NaNO_2$ (*N winogradskyi*), 690, $MgSO_4$ $7H_2O$, 40, $CaCl_2$, 0 02, NaCl, 500, KH_2PO_4, 100, and 1 ml trace element solution according to Verhagen and Laanbroek (1991) The pH of the media were adjusted to 7 7 with 1*M* NaOH before autoclavation resulting in a final pH of 7 5 5ml bromothymol blue (0 04% w/v) solution was added to the *N europaea* medium as pH indicator During the batch incubation the pH was adjusted with sterile Na_2CO_3 (1% w/v) to maintain pH 7 0-7 5 All cultures were incubated at 28°C Prior to their use in the gnotobiotic microcosms, the batch

cultures (200 ml) were centrifuged (15000 × g, 20°C) in sterile, sealed centrifugation bottles (Nalgene, Rochester, USA) Pellet were washed 3 times with an equal volume of batch medium without ammonium or nitrite The cell numbers of the washed suspension were determined microscopically by use of a Burker-Turk counting chamber The suspensions were subsequently diluted to the desired inoculation density

Pseudomonas chlororaphis ATCC 43928 was used as a representative denitrifying bacterium This species is a common soil heterotroph capable of denitrification which has been isolated from agricultural soils (Christensen and Sørensen, 1986) and was the dominant bacterial species isolated on water-agar from dune soils (DeBoer, personal communication) *P chlororaphis* also occurs in the root zone of *G maxima*, where it even tends to become dominant after amendment with nitrate (Nijburg *et al* , 1997) We used a strain only capable of reducing nitrate to nitrous oxide (Christensen and Tiedje, 1988) which is of methodological convenience Growth conditions and handling prior to inoculation of the microcosms have been described elsewhere

Experimental procedure

The calcareous river sand, originating from a floodplain from the river Rhine (Bemmel, the Netherlands), was the same as that used in previous studies (Bodelier *et al* , 1997) Sediment characteristics were pH(H_2O) 8 3, 0 66% organic matter, and 24 51% waterholding capacity Separate experiments had indicated that the bare sand did not allow for any nitrifying activity and therefore cellulose (fibrous medium, Sigma Chemical Co, St Louis, USA) was added to the sand (2% w/w) to provide an adhering surface for the bacteria Addition of cellulose led to a distinct stimulation of nitrification (data not shown) The mixture of sand and cellulose was sterilised by incubation at 200°C for 2 × 12 hours 24, autoclaved (121°C, 1 hour) microcosms were filled with 800 grams of the heated sand-cellulose mixture in a sterile flow cabinet After inundation of the sand with sterile ultra pure milliQ water (Millipore BV, Etten-Leur, the Netherlands) the microcosms were autoclaved again for 1 hour (121°C) Prior to inoculation, the sand in the systems was percolated with 1 liter of sterile ammonia oxidiser medium (see above), containing 1*mM* NH_4^+, by pouring the buffer on top of the sand and subsequently removing it via the rhizon sampling device This was done to supply the nitrifiers with substrate to establish themselves, to remove possible inhibiting compounds released during the sterilisation process, and to wash away any low molecular weight carbon compounds that might be used by *P chlororaphis* The sand was inoculated with 50 ml inoculum, containing washed cells of *N europaea* and *N winogradskyi*, leading to initial densities of 2 5×10^5 cells g^{-1} of dry sediment The microcosms were incubated at 20°C at 70-80% water holding capacity, to allow for "establishment" of the nitrifiers After two weeks the nitrate concentration in the

pore water of the systems was between 1-3 *mM*, indicating that nitrification had begun After 2 weeks the pore water in the systems was removed to reduce the amount of nitrate present At this point, half of the systems were inoculated with *P chlororaphis* (1×10^4 g^{-1} dry sediment) and 16 microcosms, half with and half without *P chlororaphis*, were planted with 4 sterile *G maxima* seedlings as described by Bodelier *et al* (1997) The sediment was flooded by the addition of sterile milli Q water All microcosms were incubated in Hereaus-Votsch HPS 1500 growth cabinets (day/night cycle 16h,20°C/8h,20°C, relative humidity 70%, photosynthetic photon flux density at plant level ± 300 μE m^{-2} s^{-1}) The air flow through the cylinders was 1 0 litre min^{-1} In the present study we also used beakers, receiving exactly the same treatment as the microcosms, to estimate the initial bacterial numbers as previously described by Bodelier *et al* (1997) The moment of the inoculation of *P chlororaphis* and the planting of *G maxima* was defined as week 1 of the experimental period Plant nutrients were added weekly, by injection into the root compartment via the microsporous sampling device, in an exponentially increasing amount through week 5 From week 5 to 8 the highest doses of the major elements (N, 3 5 mmol, P, 0 448 mmol, K, 1 312 mmol) were applied The unplanted systems did not receive any nutrients after week 5 to avoid toxic levels for the nitrifying bacteria Pore water samples from the non-root and root compartment were collected prior to nutrient addition The first 3 ml sample was discarded to avoid contamination with water from the dead space (approx 0 5 ml) of the sampler Samples were immediately transferred to 4°C and were analysed for NH_4^+, NO_3^-, NO_2^-, N_2O and pH the same day At the beginning of week 5, half of the systems was subjected to a photoperiod of 12 hours whereas, the other half was incubated with a 20 hour photoperiod Systems were randomly divided over the various treatments, with 4 replicate microcosms per treatment

Harvesting and processing of plants and soil

The systems were harvested after 8 weeks To avoid any possible bias, 1 microcosm of every treatment was processed per each of 4 harvest days Floodwater was removed and stored at 4°C for further analysis After cutting off the shoots, two stainless steel cylinders (5 and 6cm in diameter) were inserted in the non-root compartment (N) creating 3 compartments of increasing distance (cm) from the root compartment (R) N1, 0-0 5, N2, 0 5-1 0 and N3, 1 0-3 5, respectively The contents of each compartment, as well as the root compartment, were transferred to sterile glass containers for further analysis Shoot and root dry matter was determined by weighing after drying at 70°C for 2 days Moisture percentage, of thoroughly mixed sediment, was determined gravimetrically after drying at 105°C for 24 hours Mineral nitrogen content was determined in 1*M* KCl extracts (1 2 5, w/v)

Microbial parameters

Enumeration of nitrifiers and denitrifiers

The numbers of *N europaea* and *N winogradskyi* were determined using a most probable number technique according to Bodelier *et al* (1996) The technique was modified slightly in that the sand slurries were subjected to sonification (1 min, 47 kHz, Branson 2000 sonification bath) before serial dilution Numbers of *P chlororaphis* cells were determined by means of plate counting as described by Bodelier *et al* (1997)

Potential nitrifying activities

Potential nitrifying activities were taken as the slope of the nitrate + nitrite production vs time, during a 6 hour incubation period according to Verhagen *et al* (1993) In contrast to of Verhagen *et al* (1993), we incubated the slurries at 20°C instead of 25°C and stopped nitrification in the samples, withdrawn from the assay medium, by means of centrifugation (15000×g, 15 min) This proved to be as efficient as addition of 2*M* KCl (data not shown)

Potential denitrification activity

Potential denitrification activity assays were performed by the method of Tiedje (1994) using the specific assay conditions described by Bodelier *et al* (1996) The N_2O production was followed during an incubation period of 8 hours in the presence of chloramphenicol to inhibit de novo enzyme synthesis A 1ml sample was withdrawn from the assay medium for analysis of NO_2, simultaneously with each N_2O sample

Identification of infections

Colonies which were different from *P chlororaphis* were streaked out on fresh nutrient broth agar petri dishes until only one colony type appeared Isolates were identified using a gas chromatograph-software system for the analysis of whole cell fatty acid methyl ester (FAME, Microbial ID, Inc [MIDI]) profiles as described by Janse (1991)

Chemical analyses

Concentrations of NH_4^+, NO_3 and NO_2 in pore water samples, sediment extracts, nutrient solutions, floodwater, NH_3-trap and in nitrification and denitrification assays were analysed using a Technicon Traacs 800 autoanalyzer (Technicon Instr

Corp , Tarrytown, NY) Nitrous oxide in pore water samples and denitrification assays was measured by injecting headspace samples from venoject tubes into a gas chromatograph (Carlo Erba GC 6000) equipped with a Hayesep Q column (80°C), an ECD detector (nitrous oxide <100 ppmv) and a HWD detector (nitrous oxide $>$ 100 ppmv) The total amount of nitrous oxide in gas and liquid phase of the venoject was calculated using the Bunsen absorption coefficient for nitrous oxide (0 632, 20°C, 1 atm, Tiedje, 1994) The chemical composition of the plant material was analysed with dried and ground (0 5 mm) plant material as described by Troelstra (1983) and Troelstra *et al* (1995) The ratio of carboxylate and organic nitrogen was determined to provide an indication of the relative ammonium/nitrate uptake by the plants (Troelstra, 1983)

Statistical analyses

All statistical analyses were performed using the SATISTIX analytical software package (NH Analytical Software, St Paul MN) Data were checked for normality by means of the Wilk-Shapiro test Comparisons of means of plant parameters were performed by Tukey's test Effects of the presence of *G maxima* or *P chlororaphis*, the photoperiod and the distance to the root were analysed by means of a Kruskall-Wallis one way Anova followed by a parametric Anova applied on the ranked variables as described by Potvin and Roff (1993) This procedure was performed to avoid a violation of the assumption of equal variances Due to unequal sample sizes and inequality of variances it was not possible to test for interactions of treatment effects on the measured variables Correlations between the measured variables were determined by means of the Spearman-Rank correlation test Significance of the correlation coefficients were determined by using the table presented by Zar (1974)

Results

Plant parameters

Total plant dry matter production was higher for the 20-h photoperiod for all planted microcosms (Table 1) Plant yield was affected significantly by the photoperiod but not by the presence of *P chlororaphis* (Table 2) Shoot/root ratio (4 3-5 8) and relative growth rates (0 93-1 03 week $^{-1}$) did not differ between the treatments (Table 1) Analysis of RGR for main effects did however reveal a significant effect of the photoperiod (Table 2) The chemical composition of the plants was hardly affected by photoperiod or the presence of *P chlororaphis* (Table 3) Elevation of the hours of daylight only led to lower K^+ concentrations and lower NO_3 concentrations without *P chlororaphis* No significant differences were found for the carboxylate content (C-A) and the of ratio of carboxylate and organic N concentration ($C\text{-}A/N_{org}$)

Table 1 Total dry weight, shoot-root ratio (S/R) and relative growth rate (RGR) of *Glyceria maxima*, grown in gnotobiotic microcosms subjected to two photoperiods (12 vs 20 hours) and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* either with or without the denitrifier *Pseudomonas chlororaphis* Values are means (±1SE) Different letters per column indicate significant differences (Tukey's test, $p<0.05$)

Photoperiod	+/- *P chlororaphis*	Total dry weight(g)	S/R ratio	RGR (week^{-1})
12h (n=3)	-	4 73±0 64bc	5 76±0 22	0 94±0 02
12h (n=4)	+	3 68±0 36c	4 48±0 50	0 93±0 03
20h (n=2)	-	7 08±0 25a	4 27±1 16	1 03±0 05
20h (n=4)	+	6 56±0 32ab	4 62±0 34	1 03±0 02

Table 2 Effects of the presence of *Glyceria maxima*, the presence of *Pseudomonas chlororaphis*, the photoperiod and the distance to the root compartment on the measured variables in gnotobiotic microcosms The plants were subjected to different photoperiods (12 vs 20 hours) and were inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* and either with or without the denitrifier *Pseudomonas chlororaphis* The + and - between brackets indicate a promoting or supressing effect on the independent variable, respectively

	Treatment			
Variable	+/- Plant	+/- Pseudomonas	Photoperiod	Sediment layer
TDW[a]	-	NS	(+)***	-
RGR[b]	-	NS	(+)**	-
PNA[c]	(-)***	0 066	(-)**	NS
PDA[d]	(+)*	-	0 094	NS
CFU[e]	(+)***	-	(+)*	NS
MPN[f] NH_4^+-ox	NS	NS	0 091	NS
MPN[g] NO_2-ox	NS	(-)**	(-)**	NS
Mln[h] NH_4^+	(-)***	NS	(-)**	(+)**
Mln[i] NO_3	(-)***	NS	(-)***	0 081

*** $p<0.001$, ** $p<0.01$, * $p<0.05$, NS not significant When the 0 05 level was just not reached ($0.05< p >0.1$) the p value is given in the table [a] Total plant dry weight, [b] Relative growth rate of *Glyceria maxima*, [c] Potential nitrifying activity, [d] Potential denitrifying activity, [e] Numbers of colony forming units of *Pseudomonas chlororaphis*, [f] Most probable numbers of *Nitrosomonas europaea*, [g] Most probable numbers of *Nitrobacter winogradskyi*, [h] Sediment ammonium content, [i] Sediment nitrate content

Table 3 Chemical composition of *Glyceria maxima* (whole plant) grown in gnotobiotic microcosms under two photoperiods (12 vs 20 hours) The sediment in the microcosms was inoculated with *Nitrosomonas europaea* and *Nitrobacter winogradskyi* and with or without *Pseudomonas chlororaphis* Ion concentrations are expressed as meq kg^{-1} dry matter, organic nitrogen as mmol N kg^{-1} dry matter Significant differences (Tukey's test, $p < 0.05$) per row are indicated by different letters

Variable	Photoperiod/(+/-) Pseudomonas			
	12h		20h	
	Nitrifiers[1] only	+Pseudomonas[2]	Nitrifiers[3] only	+Pseudomonas[4]
K^+	1165 a	1152 a	863 b	922 b
Na^+	99 a	107 a	107 a	106 a
Ca^{2+}	270 a	249 a	309 a	264 a
Mg^{2+}	117 ab	144 a	96 b	124 ab
NH_4^+	25 a	22 a	22 a	21 a
H_2PO_4	170 a	214 a	173 a	181 a
NO_3	103 a	70 ab	9 b	10 b
Cl	611 a	586 a	600 a	596 a
SO_4^{2-}	230 a	398 a	212 a	235 a
Carboxylate (C-A)	497 a	406 a	438 a	443 a
Organic N	2944 a	2809 a	2447 a	2349 a
C-A/Norg	0 17 a	0 15 a	0 18 a	0 19 a

[1] n = 3, [2] n = 4, [3] n = 2, [4] n = 4

Microbiology

General aspects

All microcosms inoculated with *P. chlororaphis* remained free of contaminants during the complete experimental period of 3.5 months. Three of the twelve microcosms inoculated with only *N europaea* and *N. winogradskyi* remained free of any contamination, 1 in each treatment. From the remaining microcosms 3 were infected with *Acinetobacter lwoffi*, 2 with *Methylobacterium mesophilicum* and 4 contained *Pseudomonas chlororaphis*. *A. lwoffi* and *M. mesophilicum* infections did not apparently contribute to denitrification, based upon their low cell numbers and the observed pore water nitrate concentrations in the unplanted systems, and only the microcosms containing *P chlororaphis* infections were therefore discarded.

Nitrification

In Figure 2 the most probable numbers results of *N. europaea* (A,C) and *N. winogradskyi* (B,D) are presented.

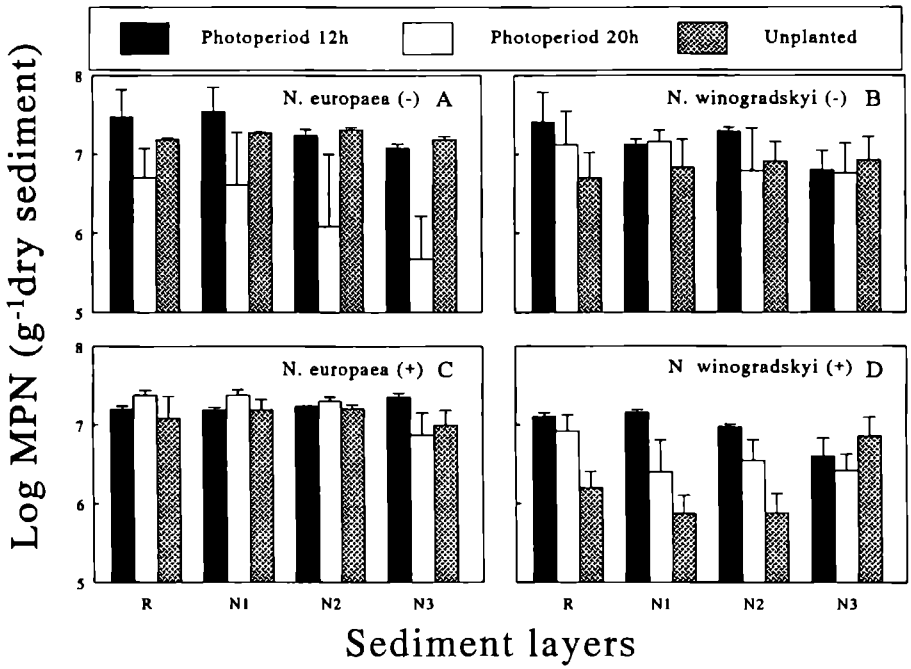

Figure 2 Most Probable Numbers of *Nitrosomonas europaea* (A, (+) *Pseudomonas chlororaphis*, C (-) *P chlororaphis*) and *Nitrobacter winogradskyi* (B (+) *P chlororaphis*, D (-) *P chlororaphis*) in 4 sediment layers of gnotobiotic microcosms with or without *Glyceria maxima*, which were subjected to two photoperiods (12 or 20 hours) The sediment layers are defined as, R root compartment and non-root layers N1, N2 and N3 at distances 0-0 5cm, 0 5-1 0cm and 1 0-3 5cm from the root compartment, respectively

Initial densities after inoculation were 1.41×10^4 and 8.31×10^4 cells g^{-1} dry sediment for *N. europaea* and *N. winogradskyi*, respectively. From the initial densities and Figure 2 it is evident that substantial growth of both nitrifying strains occurred in all treatments. The increase in numbers was 2-3 orders of magnitude. The numbers of *N. europaea* were not significantly affected by the presence of *G. maxima* or *P. chlororaphis* (Table 2). The effect of the photoperiod on numbers of *N europaea* just failed to reach the 0.05 significance level (Table 2). There was also no effect of sediment layer, including the root compartment (Table 2). *P chlororaphis* and the photoperiod had significantly negative effects on the numbers of *N winogradskyi* (Table 2).

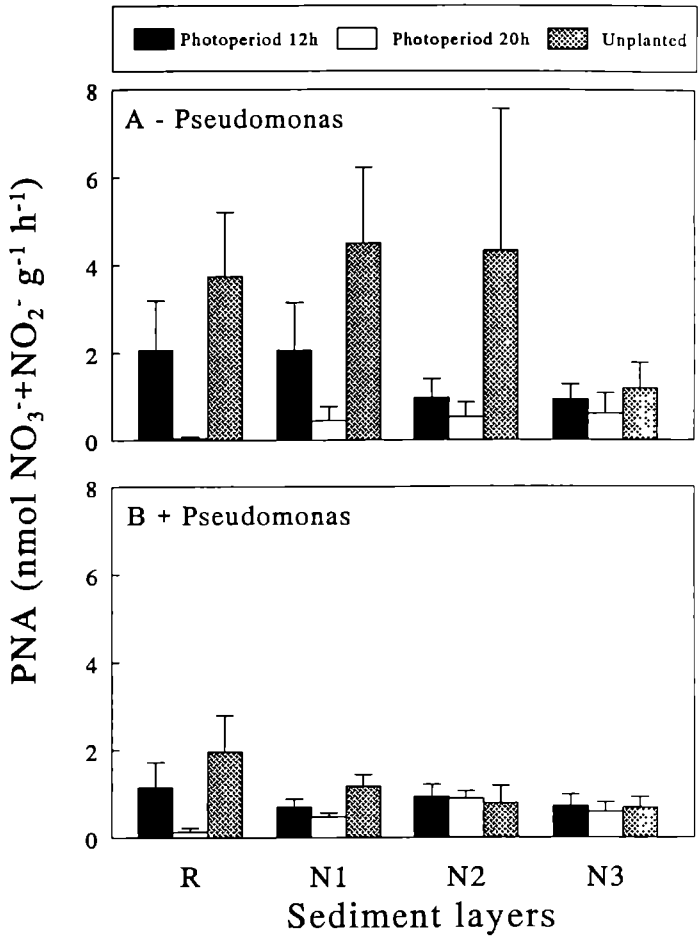

Figure 3· Potential nitrifying activities in 4 sediment layers of gnotobiotic microcosms, planted with or without *Glyceria maxima*, subjected to two photoperiods (12 or 20 hours), and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* either without (A) or with (B) the denitrifier *Pseudomonas chlororaphis* The sediment layers are defined as in Figure 2

The potential nitrifying activities in the presence of *P. chlororaphis* (Fig. 3B) were generally lower than those without *P. chlororaphis* (Fig. 3A). This effect seemed to be more pronounced in the unplanted microcosms, but the effect of *P. chlororaphis* on potential nitrifying activities just failed to reach the significance level of 0.05 (Table 2). Elongation of the photoperiod led to an almost complete suppression of nitrifying activity in the root compartment. The PNA was significantly and negatively affected by the presence of the plant and the lengthening of the photoperiod (Table 2, Fig. 3). The distance to the root compartment (Table 2) had no significant effect on nitrifying activity.

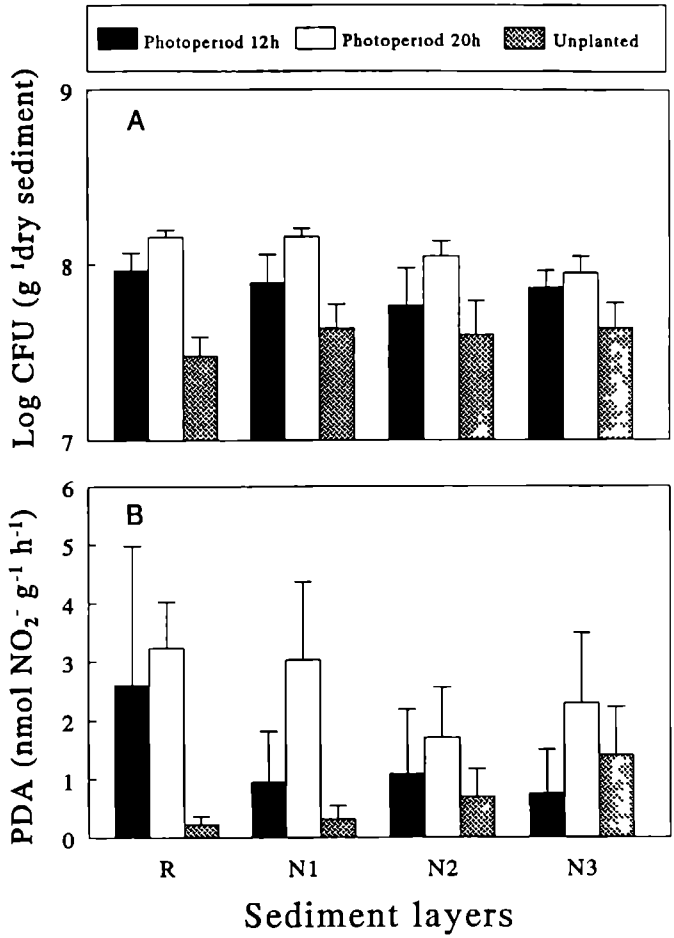

Figure 4 Numbers of *Pseudomonas chlororaphis* (A) and potential denitrifying activities (B) in gnotobiotic microcosms planted with or without *Glyceria maxima*, which were subjected to two photoperiods (12 or 20 hours), and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* The sediment layers are defined as in Figure 2

Denitrification

The initial density of *P chlororaphis*, determined after 3 days of incubation in a parallel series of glass beakers, was 1 17×10^7 CFU's g^{-1} dry sediment As we inoculated 1×10^4 cells g^{-1} dry sediment, residual substrate present in the sediment apparently allowed for cell numbers to increase by 3 orders of magnitude The final numbers reached values of up to 5×10^7 CFU's g^{-1} dry sediment in the

unplanted microcosms, and 1.4×10^8 CFU's g^{-1} dry planted sediment, occurring in the root compartment and the adjacent N1 layer of the 20 hour photoperiod (Fig. 4A). The numbers of *P. chlororaphis* were stimulated by both the presence of *G. maxima* and lengthened photoperiod (Table 2). Again, there was no significant effect of the distance from the root compartment.

The denitrifying activities are expressed as nmol NO_2^- derived from NO_3^- g^{-1} dry sediment h^{-1}. During the assays no N_2O could be detected, and the NO_2^- concentration in the assay increased linearly with time. The lowest value, 0.2 nmol NO_2^- g^{-1} dry sediment h^{-1}, was measured in the "root" compartment of the unplanted systems, while the highest value (3.2 nmol NO_2^- g^{-1} h^{-1}, Fig. 4B) was found in the root compartment of the planted systems subjected to the 20 hour photoperiod. Potential denitrifying activities were stimulated by the presence of *G maxima* (Table 2). The photoperiod and the distance from the root compartment had no effect.

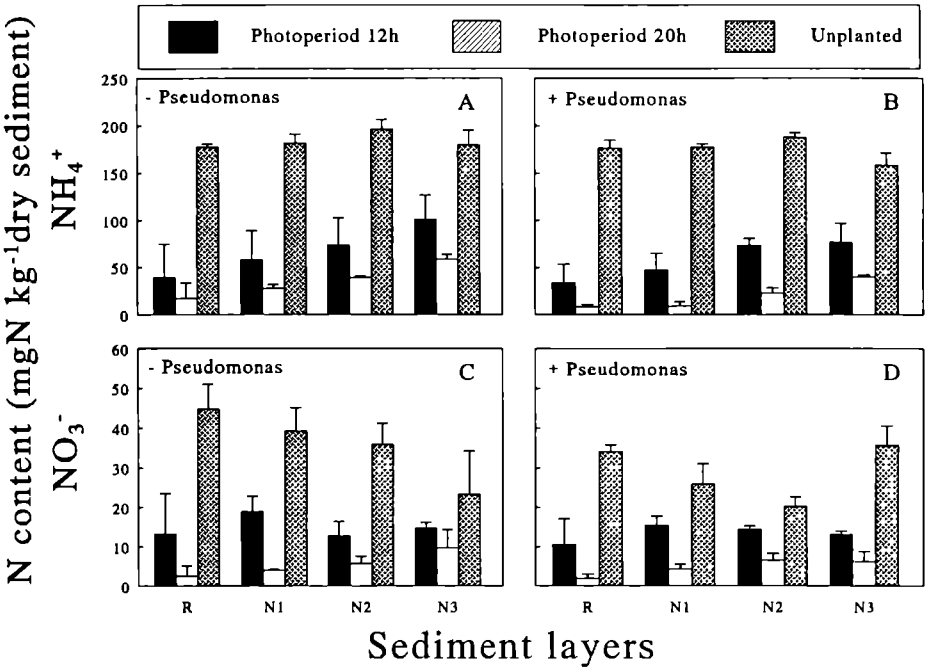

Figure 5 Sediment ammonium (A,B) and nitrate (C,D) content in 4 sediment layers of gnotobiotic microcosms planted with or without *Glyceria maxima*, which were subjected to two photoperiods (12 or 20 hours), and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, either with (B,D) or without (A,C) the denitrifier *Pseudomonas chlororaphis* The sediment layers are defined as in Figure 2

Sediment parameters

Final nitrogen content

Mineral N contents at the end of the experiment were influenced by the presence of *G. maxima,* the photoperiod and the distance to the root compartment (Table 2). The plants reduced the ammonium content compared to the unplanted microcosms (Fig. 5 A,B) and was lowest in the microcosms subjected to the 20 hour photoperiod, reaching levels of 8.5 mg NH_4^+ g^{-1} dry sediment in the root compartment of the microcosms with *P. chlororaphis*. The presence of *P. chlororaphis* had no significant effect (Table 2). From the root compartment into the adjacent non-root layers there was a gradient of increasing ammonium levels. Although nitrate concentrations were substantially lower than those for ammonium, the patterns were rather similar (Fig. 5 C,D). Nitrate concentrations were also lowered by the presence of the plant and the longer photoperiod, whereas the presence of *P. chlororaphis* had no effect (Table 2). In contrast to the ammonium, the nitrate content was not affected significantly by the distance from the root compartment (Table 2). Nitrite was only detectable in the unplanted systems and reached values of 2.0-3.8 mg NO_2 g^{-1} dry sediment (data not shown).

Pore water composition: NH_4^+, NO_3^- and N_2O

The concentrations of NH_4^+ (A,B) and NO_3 (C,D) in the pore water of the root- and non-root compartments are presented in Figure 6. Ammonium concentrations in the absence (Fig. 6A) and in the presence of *P. chlororaphis* (Fig. 6B) were very similar. In the unplanted microcosms ammonium accumulated, as expected, from nutrient addition. In the planted systems ammonium started to accumulate from week 5 on, except for the root compartment of the microcosms subjected to the 20-h photoperiod. In all microcosms, ammonium concentrations of the pore water were higher in the non-root compartment. The nitrate present, as the consequence of the preincubation period, decreased to almost undetectable levels in all microcosms, except for the unplanted systems without *P. chlororaphis* where the nitrate concentration remained in the range of 1-2 *mM*. Nitrous oxide concentrations in the microcosms due to nitrifiers alone were in the range of 0.003-3.65 μmol N_2O l^{-1} pore water (Fig. 7A) and 1.04-25.28 μmol N_2O l^{-1} (Fig. 7B) in the presence of *P. chlororaphis*. In the absence of *P. chlororaphis,* N_2O production in the planted microcosms started earlier than in the unplanted systems, where appreciable amounts were only detected after week 3. Taking the substantial variation into account, it can be stated that the N_2O levels in the root- and non-root compartments of the planted microcosms without *P. chlororaphis* stayed relatively constant through week 6. In week 7 and 8 the N_2O concentrations in the root compartment of the microcosms subjected to the 20-h photoperiod were

significantly lower compared to the non-root compartment. This was not observed with the 12-h photoperiod. In the presence of *P. chlororaphis*, N_2O concentrations were 10-12 μmol l^{-1} at the first sampling event, which was 6 days after inoculation with *P. chlororaphis*, and remained at this level through week 4. After the separation to a long and short day length the patterns became more dynamic. In the unplanted systems, N_2O levels rose to approximately 25 μmol l^{-1} pore water. The amount of N_2O in the pore water started to decrease in the planted systems, especially in the root compartments. N_2O concentrations in the root compartment of the systems subjected to the long photoperiod were significantly lower in weeks 6-8 compared to the non-root compartment. With the short photoperiod this occurred only in week 8.

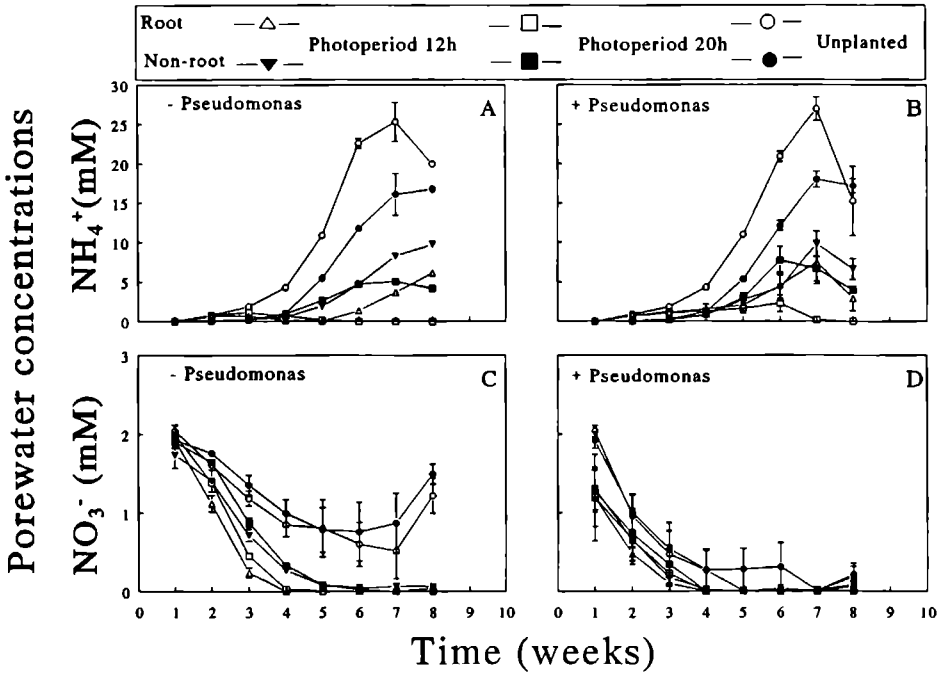

Figure 6· Porewater ammonium (A,B) and nitrate (C,D) concentrations in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, either with (B,D) or without (A,C) the denitrifier *Pseudomonas chlororaphis* Until week 5, all microcosms had been subjected to a photoperiod of 16 hours At the start of week 4, microcosms were subdivided in two series with different photoperiods of 12 and 20 hours, respectively

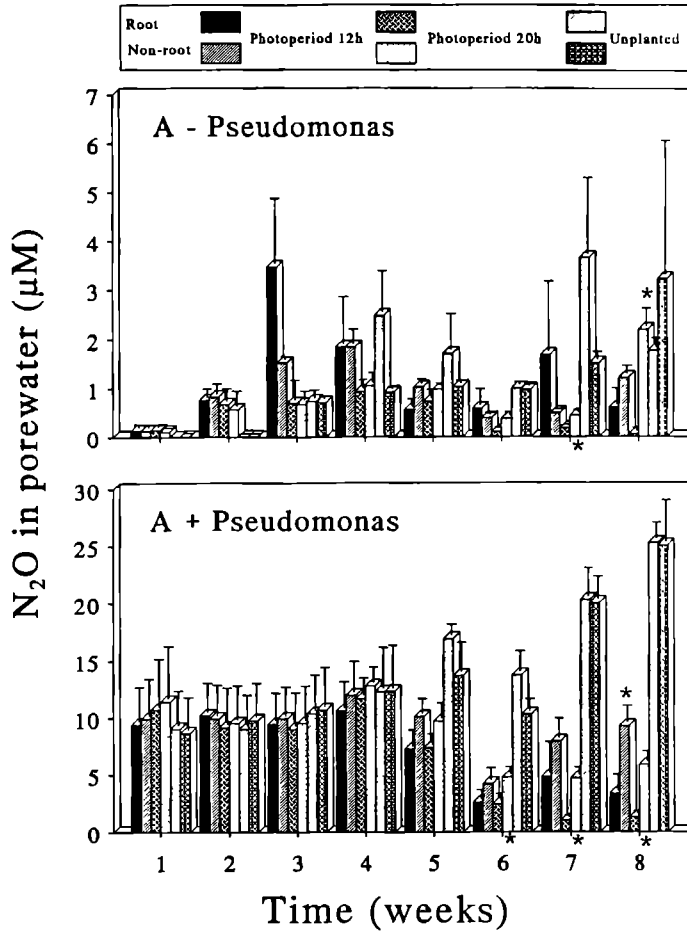

Figure 7: Porewater nitrous oxide concentrations in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, either with (B) or without (A) the denitrifier *Pseudomonas chlororaphis* Until week 5, all microcosms had been subjected to a photoperiod of 16 hours. At the start of week 4, microcosms were subdivided in two series with different photoperiods of 12 and 20 hours, respectively. Asterisks indicate significant differences between root- and non-root compartment ($p < 0.05$, two sample t-test).

Correlation of measured variables

Spearman rank correlation coefficients of the measured variables are presented in Table 4. PNA's were negatively correlated to the total plant dry weight and were positively related to ammonium and nitrate concentrations in the sediment. The numbers of *N. europaea* were negatively correlated to the numbers of

P. chlororaphis. The numbers of *N. winogradskyi* appeared to be positively linked to the denitrifying activity and were negatively correlated to the ammonium concentration. The numbers of *P. chlororaphis* were positively related to the nitrate concentration, but negatively to the ammonium content. Finally, there appeared to be a strong positive relation between the ammonium and nitrate concentration of the sediment.

Table 4 Spearman rank correlations of measured variables of gnotobiotic microcosms planted with or without *Glyceria maxima*, which was subjected to two photoperiods (12 vs 20 hours) The sediment was inoculated with the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* and either with or without the denitrifier *Pseudomonas chlororaphis*

Variable	TDW[a]	PNA[b]	PDA[c]	CFU[d]	MPN[e] NH_4^+	MPN[f] NO_2	Min[g] NH_4^-	Min[h] NO_3
PNA	-0 78** (n=13)	-	-	-	-	-	-	-
PDA	-0 03 (n=6)	-0 25 (n=40)	-	-	-	-	-	-
CFU	0 26 (n=6)	-0 22 (n=46)	-0 12 (n=40)	-	-	-	-	-
MPN NH_4^+	-0 20 (n=13)	0 09 (n=72)	0.09 (n=40)	-0 46** (n=44)	-	-	-	-
MPN NO_2	0 12 (n=13)	-0 05 (n=78)	0 33* (n=40)	-0 18 (n=48)	0 04 (n=72)	-	-	-
Min NH_4^+	-0 24 (n=13)	0 41** (n=78)	-0 08 (n=40)	-0 54** (n=48)	0 18 (n=72)	-0 26* (n=80)	-	-
Min NO_3	-0 24 (n=13)	0 54** (n=78)	-0 13 (n=40)	0 60** (n=48)	0 22 (n=72)	-0 16 (n=80)	0 82** (n=80)	-

** $p<0.01$, * $p<0.05$ [a] Total plant dry weight related to measured variables in root compartment only, [b] Potential nitrifying activity, [c] Potential denitrifying activity, [d] Numbers of colony forming units of *Pseudomonas chlororaphis*, [e] Most probable numbers of *Nitrosomonas europaea*, [f] Most probable numbers of *Nitrobacter winogradskyi*, [g] Ammonium content of the sediment, [h] Nitrate content of the sediment

Discussion

General

The modifications we applied to the gnotobiotic microcosms used in this study represented an improvement to the systems previously used, containing only *P. chlororaphis* (Bodelier *et al.*, 1997) in which the sterilisation procedure and the method of nutrient addition was apparently not effective enough. The heat

pretreatment of the sand and the addition of nutrients by means of a disconnectable flask led to a reduction of the contamination. The microcosms inoculated with *P. chlororaphis* did not become infected. However, the systems inoculated with only nitrifiers were apparently more susceptible for exposure to contaminants because of the available carbon and the slow growth of the nitrifiers.
A problem encountered in an experiment of identical design was the absolute absence of any nitrification (P.L.E. Bodelier, unpublished results). In the present study there was vigorous growth of the nitrifiers which may be explained by the wash treatment of the sediment before inoculation, thereby removing possibly inhibiting substances that have been formed during the sterilisation procedure. Another possibility is the presence of cellulose as adhering surface for the nitrifiers. Experiments with soil columns and a calcareous grassland soil (5.3% organic matter) have shown substantially higher nitrification for inoculated *N. europaea* than seen with the same mineral sand (0.66% organic matter) used in this experiment (H.J. Laanbroek and S. Gerards, unpublished results). Hence, the presence of organic adhering surfaces seems very important for nitrifier growth.

Nitrification and denitrification

The major objectives of this study were to determine the impact of *G. maxima* on the interactions between nitrifying and denitrifying bacteria with the emphasis on the potentials of nitrifiers to persist under limiting amounts of substrate (i.e. oxygen and ammonia). The increase of the carbon availability, as reflected by the numbers of *P. chlororaphis*, due to the elongation of the day length imposes a higher competitive stress upon the nitrifiers. *G. maxima* and the longer photoperiod led to an increase in the numbers of *P. chlororaphis*, as has previously been reported (Bodelier *et al.*, 1997), and the presence of *P. chlororaphis* significantly repressed growth of *N. winogradskyi,* but not of *N. europaea* (Table 2). This could be explained by the relatively poor oxygen kinetics of *N. winogradskyi* Laanbroek and Gerards, 1993) or by competition for nitrite with *P. chlororaphis*. Although the growth of *N. europaea* was not affected, the potential activities were suppressed in the presence of *P. chlororaphis*. Activities were also lowered by the plant (Table 2). Apparently, the plant limits nitrification due to ammonium uptake, as was also found by Engelaar *et al.* (1991) and Verhagen *et al* (1994). This is most obvious for the 20-h photoperiod were the plants formed more biomass and thus required more ammonium. Although one would expect the treatment variables to interact, the data did not allow for a two way ANOVA design to asses such interactions. From Table 2 it can be deduced that the photoperiod affects every measured variable probably in interaction with the other treatments. The plant apparently imposes a more important stress on the nitrifiers than *P. chlororaphis,* especially for the longer photoperiod, but for the 12-h photoperiod where ammonium is apparently not limiting, nitrification tended to be higher in the

absence of *P chlororaphis*, indicating that a part of the oxygen was consumed by the heterotroph Negative effects of *P chlororaphis* on nitrifiers due to reasons other than uptake of oxygen (e g production of antibiotics) can not be excluded Plant chemical composition (NO_3 content, carboxylate content) also indicated higher nitrate production for the 12 h photoperiod without *P chlororaphis* (Table 3) However, statistically no differences could be found between carboxylate concentrations, which in a previous experiment (Bodelier *et al* , 1997) proved to be higher for nitrate vs ammonium fed *G maxima* plants

In an attempt to elucidate the competition for oxygen, we calculated the total number of cells formed after flooding From Table 5 it can be seen that the ratio between *P chlororaphis* and *N europaea* or *N winogradskyi* in the unplanted systems hardly changed compared to day 0 These ratios do increase substantially when the photoperiod is elongated This may be due to growth inhibition of the nitrifiers due to plant N uptake However, for the 12-h photoperiod, where sufficient ammonium is available, the numbers of *P chlororaphis* increased while the nitrifiers remained at the level of the unplanted systems, indicating oxygen limitation For the 12-h photoperiod when *P chlororaphis* was not present, the nitrifiers had no competitors for oxygen and continued to multiply Hence, plant induced growth of the nitrifiers appeared only when there was sufficient ammonium and no *P chlororaphis* present The stimulation of nitrification by oxygen released by the plant was however not very substantial, as the growth in the unplanted systems using oxygen diffused from the water layer was nearly as high To form the amount of nitrate present in the unplanted systems at the end of the experiment 3 85 mmol O_2 would be needed corresponding to a flux of 8 76 mmol O_2 m^2 day^{-1} (period 56 days, surface area $7\ 85 \times 10^{-3} m^2$) Jensen *et al* (1993) measured an oxygen flux due to nitrification in the top 1 5 cm of a sediment core flooded with air saturated water of 37 6 mmol O_2 m^2 day^{-1}, demonstrating that the growth in our study might very well have been the consequence of the diffusive flux form the water layer Hence, in studies addressing the effect of oxygen releasing plants on nitrification, it is essential that the upper sediment layer receiving oxygen from the water layer should be omitted from activity measurements and bacterial counts The idea that oxygen from the water layer greatly influences the nitrification profile is supported by the fact that radial distance from the root compartment had no effect on the distribution and activity of nitrifiers It remains a possibility that the large volumes (50-100 ml) used for nutrient addition might have played a role in the distribution of plant-derived carbon and oxygen through the microcosms

Denitrification activity was enhanced by the presence of *G maxima* (Table 2) However, in the short term assays we could never detect any N_2O, while nitrite accumulated linearly An explanation might be a shortage of electron donor which can lead to incomplete denitrification (Kester *et al* , submitted) Although a substantial part was of nitrifier origin (Fig 7A), the pore water composition indicated also N_2O production by denitrification At the moment of inoculation of

Table 5 Total number of cells formed in the experimental period of 8 weeks after the day of flooding In case of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* the numbers formed were corrected for the initial densities[a] and the maximum number of cells formed in the non-flooded "activation period"[b] The numbers of *Pseudomonas chlororaphis* were only corrected the initial densities

Photoperiod	(+/-) *P chlororaphis*	*N europaea* ($\times 10^{10}$)	*N winogradskyi* ($\times 10^{10}$)	*P chlororaphis* ($\times 10^{10}$)	Ratio *P chlororaphis*/nitrifiers	
					N europaea	*N winogradskyi*
At day of flooding		0 44	0 29	0 94	2 14	3 24
Unplanted	(-)	0 59	0 39	-	-	-
Unplanted	(+)	0 70	0 40	2 67	3 79	6 65
12 hours	(-)	1 49	1 17	-	-	-
12 hours	(+)	0 71	0 68	5 53	7 79	8 13
20 hours	(-)	-0 18	0 46	-	-	-
20 hours	(+)	0 51	0 10	8 81	17 23	91 48

[a] Initial densities were determined 3 days after inoculation and were $1\ 41\times10^{4}$ g^{-1} dry sediment for *N europaea*, $8\ 41\times10^{4}$ for *N winogradskyi* and $1\ 17\times10^{7}$ for *P chlororaphis*

[b] For activation 0 8 mmol NH_4^+- per microcosm was added The number of cells formed out of this amount was calculated using the cell yields per mol N converted into nitrate at the end of the experiment in the unplanted systems without *P chlororaphis*, being 1 88 mmol The cell yields can be calculated for *N europaea* and *N winogradskyi* as $3\ 65\times10^{12}$ and $5\ 50\times10^{12}$ cells mol^{-1}, respectively Hence in the non-flooded activation period $2\ 92\times10^{9}$ and $4\ 40\times10^{9}$ cells of *N winogradskyi* and *N europaea*, respectively, could have formed per microcosm These numbers were subtracted from the total number of cells formed in the microcosms

P.chlororaphis nitrate was present from the activation period of the nitrifiers. This led to an immediate nitrite accumulation in the microcosms with *P.chlororaphis* of up to 0.9 *mM* in the pore water (data not shown), supporting the carbon shortage theory. Inhibition of the nitrite reductase by oxygen in the microcosms has certainly be taken into account when explaining the lack of N_2O production in denitrification assays.
Nitrous oxide accumulated to a level of 10 μM, and remained unchanged through week 5. The subsequent decrease of N_2O in the planted systems may have been caused by N_2O uptake by or escape via the plant, as previously reported (Reddy *et al.*, 1989b; Mosier *et al.*, 1990) or by nitrate limiting conditions due to plant nitrate uptake. The presence of nitrate in the sediment at the end of the experiment makes the latter unlikely, although pore water nitrate concentrations were low. This discrepancy between nitrate concentrations in the pore water and KCl extracts indicates a spatial variability of nitrate availability in the microcosms.
Addition of chloramphenicol to the denitrification assay medium should allow one to relate the denitrification activity to nitrate production. However, no relation could be found between the potential nitrifying activity or nitrate content of the sediment and denitrification activity (Table 4). Apparently factors other than nitrate production influenced the detected enzyme levels. The availability of carbon may be the key factor as seen by Ambus (1993), where addition of glucose stimulated denitrification 2.5 fold in streamside soil while addition of nitrate had no effect. Dendooven and Anderson (1995) have also demonstrated that even under aerobic conditions a high nitrate reductase potential was maintained enabling denitrifiers to react on the presence of carbon and anoxia.

Synthesis

To our knowledge there are no reports available which have addressed the issues of the present study by means of gnotobiotic systems. Experiments with pure cultures of nitrifiers in pre-sterilised sand or soil in the presence of a plant have only been performed under drained conditions. The numbers of nitrifiers observed in the present study were comparable to those found in gnotobiotic sand columns planted with *Pisum sativum* L. and continuously fed with ammonium (Hynes and Nelson, 1990), where ammonium and oxygen are not limiting. Even under flooded conditions, it appears that the availability of ammonium, and not oxygen, may be more important, as indicated by the positive correlation between mineral ammonium content and potential nitrifying activities (Table 4). The suppression due to ammonium uptake by the plant with 20 hours of daylight might be comparable to the field in summer. The maximal vegetation may be responsible for the observed repression in nitrification in the root zone of *G. maxima* (Bodelier *et al.*, 1996). The 12-h photoperiod might reflect the situation in early spring, where the initially lower ammonium demand of the plants allows for nitrifiers to become active. But

even under these reduced photosynthetic periods nitrifiers have to compete for oxygen with heterotrophic bacteria such as *P chlororaphis* in our experiment In addition of the decisive role of ammonium, this study reveals that the survival of nitrifiers in the root zone of aerenchymatous plants does not depend on their competitive abilities Nitrification activity was already repressed by *P chlororaphis* in both the unplanted systems and the planted columns at the 12-h photoperiod It is likely that increasing the available carbon would lead to an even more severe suppression of nitrification as was demonstrated for biofilms (Zhang *et al* , 1994, Ohashi *et al* , 1995) and sludge reactors (Hanaki, 1990) Nevertheless, when ammonium is available, nitrifiers are able to persist and to function at low oxygen conditions Unravelling the mechanisms underlying the persistence of nitrifiers in adverse habitats, without the possession of competitive abilities, is worthwhile since these mechanisms in soil or sediments may be ecologically of greater importance than the rapid and efficient growth displayed by "laboratory" bacteria

Chapter 5

Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptations of ammonia-oxidising bacteria to low oxygen or anoxic habitats.

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Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptations of ammonia-oxidising bacteria to low oxygen or anoxic habitats.

Abstract

Oxygen-releasing plants may provide aerobic niches in anoxic sediments and soils for ammonia-oxidising bacteria The oxygen releasing aerenchymatous emergent macrophyte *Glyceria maxima* had a strong positive effect on numbers and activities of the nitrifying bacteria in its root zone in spring and early summer The stimulation of the aerobic nitrifying bacteria in the freshwater sediment, ascribed to oxygen release by the roots of *G maxima*, disappeared in late summer Numbers and activities of the nitrifying bacteria were positively correlated, and a positive relationship with denitrification activities also was found To assess possible adaptations of ammonia-oxidising bacteria to low oxygen or anoxic habitats a comparison was made between the freshwater lake sediment and three soils differing in the oxicity profiles Oxygen kinetics and tolerance to anoxia of the ammonia-oxidising communities from these habitats were determined The apparent K_m values for oxygen of the ammonia-oxidising community in the lake sediment were in the range of 5-15 μM, which was substantially lower than the K_m values for oxygen of the ammonia-oxidising community from a permanently oxic dune location Upon anoxic incubation, the ammonia-oxidising communities of dune, chalk grassland and calcareous grassland soils lost 99, 95 and 92% of the initial nitrifying capacity respectively In contrast the ammonia-oxidising community in the lake sediment started to nitrify within one hour upon exposure to oxygen at the level of the initial capacity

It is argued that the conservation of the nitrifying capacity during anoxic periods and the ability to react instantaneously to the presence of oxygen are important traits of nitrifiers in fluctuating oxic/anoxic environments such as the root zone of aerenchymatous plant species

Introduction

Flooded soils and sediments are generally anoxic, except for the upper few millimeters This is due to a reduced diffusion of oxygen in water compared with air in combination with oxygen-consuming processes (Ponnamperuma, 1984) The oxidation of ammonia to nitrite and nitrate, by ammonia- and nitrite-oxidising bacteria, can only occur in the presence of oxygen and will thus be restricted to the marginal oxic parts of flooded soils and sediments However, a large number of plants which grow in anoxic soils or sediments, contain aerenchymatous tissue by which they establish a gas space continuum between the atmosphere and the root tissue (Armstrong *et al* , 1994, Laan *et al* , 1989a) The aerenchymatous tissue, which is one of the possible adaptations to anoxic root environments in a vast array of plants (Justin and Armstrong, 1987, Blom *et al* , 1994, Blom and Voesenek, 1996), provides the roots with oxygen to maintain root respiration A part of the oxygen, which diffuses to the roots, leaks into the root zone and thus elevates redox conditions preventing the build up of phytotoxic reduced compounds (Fe^{2+},

Mn^{2+}, S^{2-}) in the rhizosphere (Flessa and Fisher, 1992; Armstrong *et al.*, 1994). Hence, the root zone of aerenchymatous plants may form a niche for the aerobic ammonia- and nitrite-oxidising bacteria which oxidise ammonia to nitrite and nitrate. The produced nitrate can subsequently be used by denitrifying bacteria in the adjacent anoxic sediment or soil, which reduce it to molecular nitrogen or nitrous oxide (Tiedje, 1988), leading to nitrogen loss from the ecosystem. The availability of oxygen for nitrifiers might depend on the developmental stage of the plant in the growing season. It has been established that radial oxygen loss from aerenchymatous roots is linked to the respiratory activity of the roots which varies with temperature and age of the root (Moorehead and Reddy, 1988; Brix and Schierup, 1990; Caffrey and Kemp, 1991).

Although *in situ* nitrification in the rhizosphere of aerenchymatous plants has never been reported, a number of studies have indirectly demonstrated, a stimulation of the nitrification process by oxygen-releasing plants (Hansen and Ostergaard Andersen, 1981; Christensen and Sørensen, 1986; Reddy *et al.*, 1989b; Both *et al.*, 1992a; Engelaar *et al.*, 1995). However, none of these studies gives any information on the survival mechanisms of the nitrifiers in those oxygen-limited environments, where they probably have to compete for the available oxygen with heterotrophic bacteria as well as chemical oxidation processes. The competitive ability for a limiting substrate is defined as the ratio of the maximum consumption capacity (i.e. V_{max}) and the affinity constant (i.e. K_m) (Healy, 1980). Nitrifying bacteria are supposed to be poor competitors for oxygen due to their low V_{max} and high K_m values, respectively, compared to heterotrophs (Sharma and Ahlert, 1977; Prosser, 1989). Thus, nitrifying activity in the rhizosphere of aerenchymatous plants is only possible when there is a surplus of oxygen, assuming that ammonium is not limiting and that there is no spatial separation between nitrifiers and heterotrophs. Another possibility is that nitrifying bacteria have the ability to adapt by yet unknown means to conditions of limited oxygen supply.

The goals of this study were to assess whether oxygen-releasing plants can provide a niche for nitrification and subsequent denitrification and to test whether nitrifying bacteria are adapted to the life in sub-oxic or periodically anoxic habitats. To this end, the numbers and potential activities of the nitrifying and the denitrifying bacteria inside and outside a *Glyceria maxima* (Hartm.) Holmb. stand were followed in the littoral zone of a freshwater lake sediment during the course of one year. *G. maxima* was chosen because this plant, which is a very common plant in the Netherlands, has been indicated to stimulate nitrifying bacteria in its rhizosphere (Both *et al.*, 1992a). Moreover, *G. maxima* is highly aerenchymatous (Smirnoff and Crawford, 1983) and the inability of its roots to survive anoxia predicts continuous oxygenation of the root tissue and oxygen leakage to the rhizosphere (Brändle and Crawford, 1987; Rees *et al.*, 1987). The availability of information on the composition of the nitrate-reducing bacterial community in the *G. maxima* root zone (Nijburg *et al.*, 1997) was another argument for the choice of

this plant species
Oxygen kinetics and tolerance to anoxia of the nitrifying bacteria, in the presumably oxygen-limited sediment and three soil types differing in oxygen availability, were determined to asses possible adaptations of the ammonia oxidisers to sub-oxic/anoxic conditions

Materials and Methods

Site description and sampling

For the study of the effect of emergent macrophytes on nitrification and denitrification a *Glyceria maxima* (Hartm) Holmb (reed sweet grass) stand in Lake Drontermeer, near the township of Elburg (The Netherlands, 52°58'N, 5°50'E), was selected This stagnant freshwater lake location was chosen because a stable population of *G maxima* has been present here for at least 10 years In the sampled area *G maxima* represented 100% of the vegetation present Five intact sediment cores (25 cm in depth, 10 cm in diameter) were collected inside the *G maxima* stand and another five in the bare sediment outside the *G maxima* stand during the period from May 1994 trough May 1995 The cores were transported to the laboratory in PVC cylinders with water from the lake on top Prior to any further measurements, the top 5 cm of the cores were removed to exclude the nitrifying bacteria which might have received oxygen by diffusion from the water layer From the remaining part of the cores, the rooted fraction (15 cm) was separated from the roots, sieved (4 mm), homogenized and stored (4°C) prior to use for measurements All measurements performed on the sediment/soil as described below were completed within 3 days after sampling For dry weight determinations roots were dried for 48 hours at 70°C
To detect possible adaptations of the nitrifying bacteria in the sediment to sub-oxic conditions, a number of soils which differ in oxygen availability were selected for comparison As a "control" soil (permanently oxic) sand from the top of a coastal dune (Meijendel, near The Hague, the Netherlands, 52°08'N, 4°19'E) was collected Two locations were sampled as "intermediate soil types" (= periodically or partially subjected to low oxygen conditions) Soil from a chalk grassland in The Gerendal (50°5'N, 5°54'E) in the south of the Netherlands was collected because the resident nitrifying community at that location is partially subjected to anoxia This soil, containing a high percentage of loess, will contain anoxic micro sites due to the high moisture percentage (Table 1), which is also indicated by high denitrification enzyme activities (data not shown) A calcareous grassland situated in a river levee near the village of Brummen
(the Netherlands)(52°5'N, 6°9'E) was chosen as a "periodically anoxic" location as it is flooded on an irregular basis In the winter prior to sampling, this grassland had been flooded for a substantial period of time From these locations, the top 5

cm of 5 randomly selected plots was sampled by means of a grass-plot sampler. This was done by placing a frame, consisting of 16 squares (225cm^2 each), randomly at 5 positions on top of the soil. At each position 2 sets of 16 cores (5cm in depth, 2.5cm in diameter) were taken, pooled and regarded as one replicate. The soil collected from these locations also was separated from roots, sieved (4mm) and homogenised. The moisture content was determined as weight loss after drying (24h, 105°C) and organic matter content as loss on ignition (4h, 550°C). The pH was measured in 1:2.5 (w/v) soil/sediment-water slurries after 2 hours of shaking. Ammonium, nitrite and nitrate content of the soils and sediment was determined by extraction with 1*M* KCl (1:2.5 w/v) using a Technicon Traacs 800 autoanalyer. Some descriptive parameters, important for nitrification, for all sampled locations are presented in Table 1.

Table 1 Descriptive parameters of all sampling sites important for nitrification (May 1995)

Sediment/ soil type	Moisture% (w/w)	Temp [1] (°C)	pH H_2O	Org matter%	NH_4^+ (mg N kg^{-1}dry soil/ sediment)	NO_2	NO_3
Calcareous grassland	0 08	15	7.92	5 32	2 24	ND[2]	4 34
Chalk grassland	0 30	16	7 87	12.53	4 48	ND	5 88
Dune top	0 01	19	7.90	2 50	1 96	ND	2 94
Lake sediment							
root zone	0 28	19	7 56	2 44	2 31	ND	ND
bare	0 33	19	7 90	4 11	14.46	ND	ND

[1] Temp. = temperature at 5 cm depth.
[2] ND = not detectable

Redox measurements

To get an indication regarding microbial oxidation processes that can occur in the root zone of *G. maxima* and in the bare sediment at lake Drontermeer, redox potentials were determined as described by Clevering and van der Putten (1995). Immediately upon arrival in the laboratory, redox potentials were determined at 10 cm depth in the intact sediment cores by using a platinum electrode, placed in the center of the core, and a calomel reference cell. The potentials were transformed into redox potentials by adding the potential of the reference cell (+242 mV).

Enumeration of nitrifying bacteria

Ammonia- and nitrite-oxidising bacteria were enumerated by a Most Probable Number (MPN) technique described by Verhagen and Laanbroek (1991) Twenty grams of moist sediment/soil were shaken for 2 hours (180 rpm, 20°C) in a buffer solution containing (grams per liter) NaCl, 8 5, Na_2HPO_4 $2H_2O$, 1 34, and NaH_2PO_4, 0 35 Sub-samples (0 25 ml) from the suspensions were serially diluted in tenfold steps in sterile microtiter plates (tissue culture cluster, 24 wells, Costar, Cambridge, United Kingdom) containing growth medium for ammonia- and nitrite-oxidising bacteria, respectively, as described by Verhagen and Laanbroek (1991) Seven replicates were made per dilution, 1 dilution series was not inoculated with a soil/sediment sub-sample and was regarded as a negative control After an incubation period of 8 weeks at 28°C, ammonium oxidation was scored in each well by checking for the presence of nitrite or nitrate by adding Griess Ilosvay reagents (Schmidt and Belser, 1994) and, subsequently Zinc powder Nitrite oxidation was detected by addition of the Griess Ilosvay reagents to check for the disappearance of the added nitrite Most probable numbers of nitrifying bacteria were obtained from statistical tables using a computer program as previously described by Verhagen and Laanbroek (1991)

Determination of potential ammonium-oxidising activity and oxygen kinetics

Potential ammonium-oxidising activity and oxygen kinetics of ammonia-oxidising bacteria were determined using sediment/soil suspensions in the presence of excess ammonium and different oxygen concentrations Flasks (500 ml) containing 20 g of fresh sediment/soil, 0 15 g $CaCO_3$ and 50 ml of assay medium (grams per liter) $(NH_4)_2SO_4$, 0 33, K_2HPO_4, 0 14, KH_2PO_4, 0 027, pH=7 5) were flushed with N_2 (purity > 99 9%) to remove all oxygen Different oxygen concentrations were achieved by introducing oxygen (purity > 99 5%) into the head space of the flasks and subtracting an equivalent amount of gas from the head space to maintain atmospheric pressure Sediment/soil from each replicate core was incubated with 11, 7, 5, 3, 2 and 1% of oxygen (v/v) in the head space of the flasks which corresponds to oxygen concentrations in the assay medium of ± 100, 70, 50, 40, 15, 5μM O_2 respectively Pilot experiments revealed maximum ammonium oxidation rates at an oxygen concentration of 10% (v/v) in the head space of the flasks (data not shown) The flasks were incubated on a rotary shaker (180 rpm, 20°C) in horizontal position to optimize the transfer of oxygen from the gas to the liquid phase The production of nitrate and nitrite during 8 hours of incubation was monitored by periodic withdrawal of 1 ml samples by means of a syringe The samples were centrifuged (15 000 × g, 15 min) to remove soil/sediment particles and nitrifying bacteria Centrifugation proved to stop nitrification as efficiently as the addition of 2M KCL (data not shown) The supernatant was analysed for nitrate

and nitrite content, as described above, within 2 days after sampling Samples were stored at 4°C prior to analysis The ammonium-oxidising activity was calculated from the slope of the linear progression curve of nitrate + nitrite production versus time Nitrate + nitrite production during the first 8 hours of incubation was always linear ($R^2 > 0\ 90$) These activities were stochiometrically converted to oxygen consumption activities (ammonium oxidation rate x 1 5) The apparent half saturation constant for oxygen (K_m) and the maximum oxygen consumption rate (V_{max}) were derived from plots of oxygen consumption activities versus oxygen concentration in the liquid phase by means of the computer program Enzpack (version 2 0, P A Williams,Bangor, Wales) using the direct linear method (Eisenthal and Cornish-Bowden, 1974) Oxygen concentration in the head space was determined with a gas chromatograph (Carlo Erba GC 6000) equipped with a HWD detector and a molsieve 5Å column, operated at 80°C with Helium as a carrier gas Oxygen concentration in the liquid phase was calculated using the Bunsen absorption coefficient for oxygen (0 0311, 20°C, 1 atm) (Tiedje, 1994) in combination with the measured oxygen concentrations in the head space of the flasks

Denitrification activity assay

Denitrification activity assays were performed by the method of Tiedje (1994), in flasks (500 ml) containing 20 g of fresh sediment and 50 ml of the following medium (grams per liter), KNO_3, 1 01, K_2HPO_4, 0 14, KH_2PO_4, 0 027, Glucose H_2O, 1 98, chloramphenicol, 0 1 The flasks were flushed with N_2 (purity > 99 9%) After addition of 10kPa acetylene, to inhibit nitrous oxide reduction, the flasks were incubated in horizontal position on rotary shaker (180 rpm, 20°C) Nitrous oxide production was measured by injection of head space samples into a Gas chromatograph (Carlo Erba GC6000) equipped with a ECD detector and Hayesep Q column (80°C) for gas separation Denitrification activity was calculated from the slope of the linear progression curve of nitrous oxide concentrations during 8 hours of incubation Accumulation of nitrous oxide in that period was always linear ($R^2 > 0\ 90$)
The applied concentration of chloramphenicol effectively inhibited the *de novo* enzyme synthesis (data not shown)

Effect of anoxia on nitrification

To investigate the effect of prolonged anoxic conditions on nitrification activity, flasks (100 ml) containing 10 g of fresh sediment/soil and 25 ml of the medium used for the nitrification assay, described above, were flushed with N_2 (purity > 99 9%) and incubated at 20°C in the dark for 0,1,2,3 and 4 weeks After anoxic incubation, the flasks were flushed with air and subsequently incubated on a rotary

shaker (180 rpm, 20°C) Nitrification activity was determined by following nitrate and nitrite production during an 8 hour incubation period

Statistical analysis

All statistical tests were performed with the aid of the STATISTIX analytical software package (NH Analytical Software, St Paul MN 55117, USA) All data were checked for normality with the Wilk-Shapiro test Pairwise comparison of means, root zone versus bare sediment, were performed by means of the Two sample t-test which provides for inequality of variances as described by Snedecor and Cohran (1973) Multiple comparisons of means were performed with Tukey's test In the case of inhomogeneity of variances, determined with Bartlett's test after a one way ANOVA, data were log transformed In the case of inhomogeneity of variances after transformation, separate t-tests were performed with a Bonferonni correction of the significance level for the number of comparisons Significance of correlation coefficients were determined by means of a table presented by Snedecor and Cochran (1973)

Results

Redox potentials

As can be seen in Figure 1, the presence of the plant resulted in an elevation of the redox level Redox potentials in the root zone of *G maxima* were in the range of -73 to +112 mV and were significantly higher than the values in the bare sediment (-163 to -23 mV) The values found in the root zone were indicative of iron reduction whereas the values observed in the bare sediment indicated the reduction of sulphate (Ponnamperuma, 1984)

Ammonium content of the sediment

Except for June, 1994, the ammonium content was always higher in the bare sediment compared to the root zone (Table 2) Ammonium was never depleted at any sampling date Ranges found were 1 6-4 5 mg N kg^{-1} dry sediment for the root zone and 6-15 mg N kg^{-1} in the bare sediment

Effect of G. maxima on nitrifying bacteria

The numbers of ammonia- (Fig 2a) and nitrite- (Fig 2b) oxidising bacteria were significantly higher in the root zone of *G maxima* in the spring and summer of 1994 as compared to the bare sediment
Over the course of the growing season, the differences in size of the nitrifying

community between the root zone and the bare sediment disappeared. As soon as the shoots of the plants started to emerge in the spring of 1995, higher numbers could once again be found in the root zone, except for the numbers of nitrite oxidisers in May of 1995. The nitrite-oxidizing bacteria substantially outnumbered the ammonia oxidisers both in the root zone and the bare sediment for all samples. The ammonium-oxidising activities (Fig.3) reflected the pattern observed with the most probable numbers.

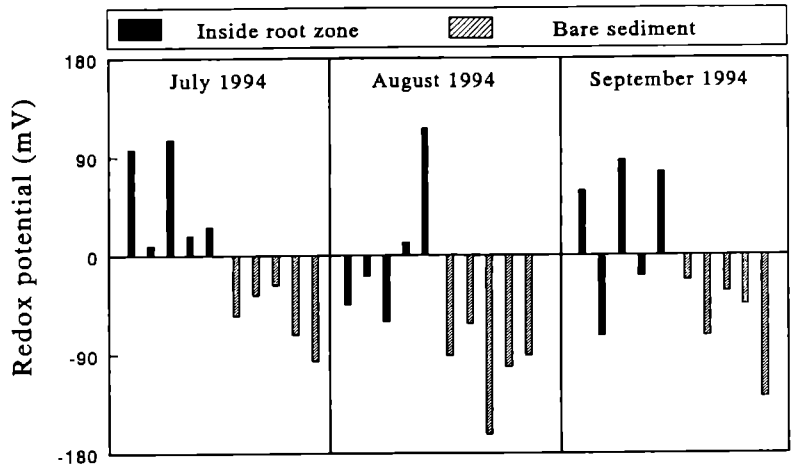

Figure 1· Redox potentials in the root zone of Glyceria maxima and in the bare sediment of Lake Drontermeer at a depth of 10 cm The presented values are the recordings for all replicate cores of July, August and September of 1994

Tabel 2. Ammonium content of sediment cores sampled in the root zone of *Glyceria maxima* and in the bare sediment at Lake Dontermeer in 1994 and 1995 Values represent means (±1SE) of 5 replicate cores. Significant differences in ammonium content between the root zone and the bare sediment are indicated by different letters (Two sample t-test, n=5, p<0 05)

		Ammonium content (mg N kg^{-1} dry sediment)	
Month/year of sampling		Inside root zone	Bare sediment
May	1994	4 54±0.58a	14 97±2 59b
June		3 53±0 50a	6 01±1 65a
July		2 50±0 31a	7 34±1 79b
August		4 07±1.11a	8 72±1 12b
September		1.58±0 17a	13 71±1 61b
November		2 18±0 22a	12.25±2 20b
March	1995	2 93±0 30a	13 02±2 83b
April		3 89±0 48a	13 15±2 43b
May		2 31±0.29a	14 46±3 15b

Oxygen kinetics of the ammonia-oxidising community

The apparent half saturation constant for oxygen (K_m) of the ammonia-oxidising community in the Lake Drontermeer sediment varied between 5 and 15 μM and did not differ significantly between the bare sediment and the sediment from the root zone (Table 3). The calculated specific affinity or substrate-sequestering ability for oxygen of the ammonia-oxidising community, which is defined as the ratio between maximum oxygen consumption rate and half saturation constant (V_{max}/K_m), is also shown in Table 3. The specific affinity for oxygen of the ammonia-oxidising community was highest in spring and only differed from the bare sediment in May of 1994 and 1995. The fluctuations in specific affinity in the root zone were the consequence of the differences in maximum oxygen-consuming potentials of the ammonia-oxidising community rather than changes in apparent K_m for oxygen, which did not change significantly during the sampling period.

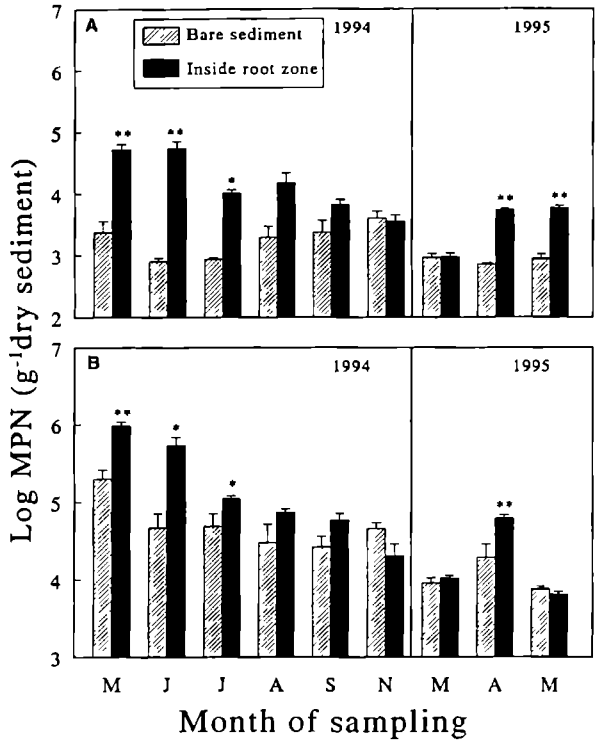

Figure 2: Most probable numbers of the ammonia- (A) and nitrite-oxidising (B) bacteria in sediment cores sampled inside the root zone of *Glyceria maxima* and in the bare sediment at Lake Drontermeer in 1994 and 1995. Bars represent means (± 1SE) of 5 replicate cores. Significant differences between the numbers in the root zone and the bare sediment are indicated by asterisks (Two sample t-test, n=5, ** $p<0.01$, * $p<0.05$).

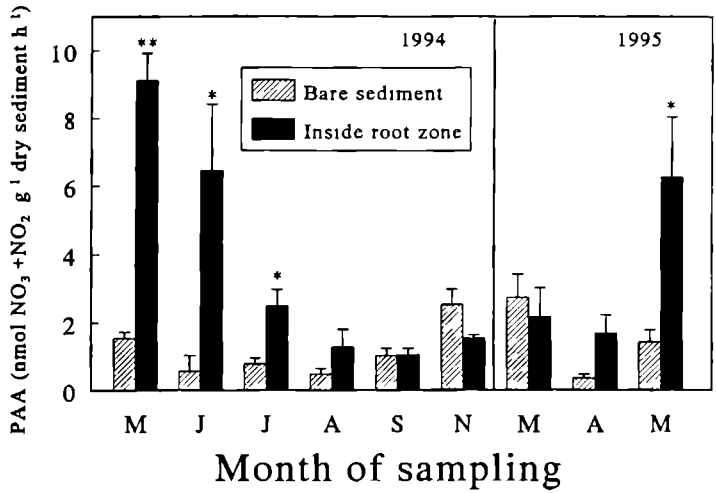

Figure 3 Potential ammonium-oxidising activities in sediment slurries of cores sampled in the root zone of *Glyceria maxima* and in the bare sediment at Lake Drontermeer in 1994 and 1995 Bars represent means (± 1SE) of 5 replicate cores Significant differences between the activities in the root zone and in the bare sediment are indicated by asterisks (Two sample t-test, n=5, ** p<0 01, * p<0 05)

Tabel 3 Apparant half saturation constants (K_m) and specific affinities (ml g^{-1} of dry sediment h^{-1}) for oxygen of the ammonia-oxidising community of sediment cores sampled in the root zone of *Glyceria maxima* and in the bare sediment at Lake Dontermeer in 1994 and 1995 Values represent means (±1SE) of 5 replicate cores Asterisks indicate significant differences between the root zone and the bare sediment (Two sample t-test, n=5, p<0 05)

Month/ year of sampling		Kinetics of oxygen consumption			
		Inside root zone		Bare sediment	
		K_m O_2 (μM)	Spec aff (ml g^{-1} h^{-1})	K_m O_2 (μM)	Spec aff (ml g^{-1} h^{-1})
May	1994	11 18±2 27	1 42±0 27*	7 20±1 65	0 43±0 27
June		12 95±2 72	0 71±0 18	5 34±2 10	0 34±0 13
July		11 24±2 47	0 35±0 06	8 64±1 86	0 18±0 07
August		7 23±1 02	0 45±0 22	10 85±2 75	0 08±0 02
September		8 04±3 36	0 32±0 08	6 05±1 33	0 36±0 15
November		7 36±1 10	0 33±0 06	11 11±4 25	0 68±0 28
March	1995	13 61±5 20	0 28±0 09	14 74±4 94	0 28±0 10
April		6 44±1 92	0 72±0 37	4 50±1 61	0 16±0 03
May		9 06±1 53	1 01±0 23*	8 49±1 31	0 27±0 03

Denitrification activity

The results of the denitrifying activity measurements that were determined in the presence of chloramphenicol to inhibit *de novo* enzyme synthesis, are presented in Figure 4. No measurements were performed in May 1994. The denitrification activity was stimulated by *G. maxima* in early summer. This effect disappeared again as the growing season progressed. In the fall of 1994 and in March of 1995, the denitrification activity even tends to be higher in the bare sediment. The pattern in 1994 was very similar to the numbers and activities of the nitrifying bacteria. The stimulation of nitrification by *G. maxima* in the spring of 1995 was however not reflected in the denitrifying activity.

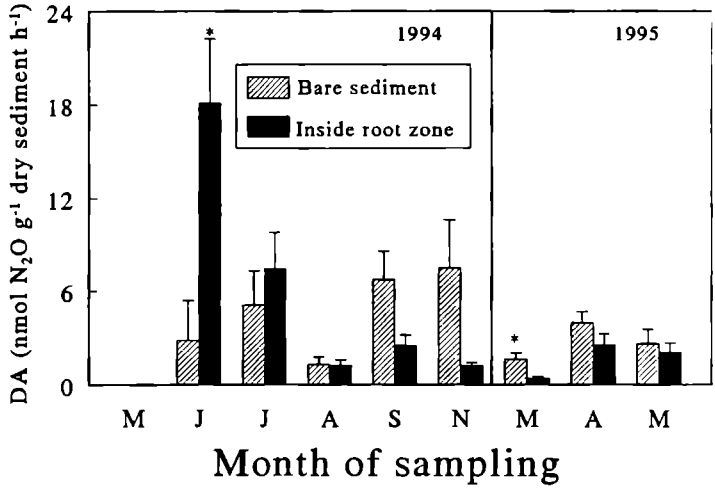

Figure 4 Denitrification activities (DA) in slurries from sediment cores sampled in the root zone of *Glyceria maxima* and the bare sediment at Lake Drontermeer in 1994 and 1995 Bars represent means (±1SE) of 5 replicate cores Significant differences between activities in the root zone and the bare sediment are indicated by asterisks (Two sample t-test, n=5, ** $p<0.01$, * $p<0.05$)

Correlations of the measured variables

The correlation coefficients of the measured variables of Lake Drontermeer are presented in Table 4. Nitrifying activities and numbers were significantly correlated with denitrification activities in the root zone of *G. maxima* and in the bare sediment. The correlation between the numbers of ammonia- and nitrite-oxidising bacteria with denitrification activities was clearly evident Nitrification activities and numbers were only correlated in the root zone. Numbers of ammonia- and nitrite-oxidising bacteria were highly correlated in the root zone, whereas this relationship was much weaker in the bare sediment. The ammonium content of the

sediment was only significantly correlated with the numbers of ammonia- and nitrite-oxidising bacteria in the root zone. The root biomass of *G. maxima* showed no significant correlation with any of the measured variables.

Table 4 Pearson correlation coefficients of measured variables of soil cores sampled in the root zone of *Glyceria maxima* and in the bare sediment ( ).

Variable	[1]PAA	[2]DA	[3]MPN NH_4^+	[4]MPN NO_2	[5]Min NH_4^+
PAA	-	-	-	-	-
DA	0.52**	-	-	-	-
	(0 46**)	-	-	-	-
MPN NH_4^+	0 47**	0 78**	-	-	-
	(0 22)	(0.37*)	-	-	-
MPN NO_2	0 51**	0 84**	0 94**	-	-
	(0 11)	(0 53**)	(0 32*)	-	-
Min.NH_4^+	0 22	0.26	0.31**	0.32**	-
	(0 25)	(0 19)	(0 21)	(-0 11)	-
Root biomass (dry weight)	0 15	-0 16	-0.25	-0 23	0 06

[1] Potential ammonium-oxidising activity, [2] Denitrification activity, [3] Most Probable Numbers of the ammonia-oxidising bacteria, [4] Most Probable Numbers of the nitrite-oxidising bacteria, [5] Mineral nitrogen available in the form of NH_4^+
Df = 43 for PAA, MPN, Min NH_4^+ and root biomass, Df = 38 for DA, * $p<0\ 05$, ** $p<0\ 01$

Characteristics of the ammonia-oxidising community of Lake Drontermeer and soils with different oxygen availabilities.

Oxygen kinetics

The oxygen kinetics of the ammonia-oxidising community from the root zone of *G maxima* and from soils which differ in oxygen availability are presented in table 5. The apparent K_m for oxygen of the ammonia-oxidising community of the dune top was substantially higher than that of all other locations. The maximum oxygen consumption rate of the ammonia-oxidising community was lowest in the lake sediment, resulting in a specific oxygen affinity value equal to that of the dune top. The specific affinities of the dune top and lake sediment communities appeared to be significantly lower than that of the ammonia-oxidising communities of the two grassland locations.

Table 5 Oxygen kinetics of the ammonia-oxidising community at locations which differ in oxygen availability

Sediment/soil type	Km[1] (μM O_2)	Vmax[2] (nmol O_2 g^{-1} h^{-1})	Spec aff [3] (ml g^{-1} h^{-1})
Lake sediment (*root zone*)	9 06a	9 43a	1 01a
Calcareous grassland	11.29a	65 02b	6 23b
Chalk grassland	11 02a	63 98b	6 14b
Dune top	28 88b	30 70c	1 09a

[1]K_m = apparent half saturation constant for oxygen, [2]V_{max} = maximum oxygen consumption rate, [3]Spec aff = specific affinity, calculated as V_{max}/K_m
Different letters indicate significant differences between means ($p<0.05$, $n=5$, except for the chalk grassland location where $n=4$). Apparent K_m and specific affinity data were analysed by Tukey's test after logarithmic transformation to achieve homogeneity of variances The V_{max} data were analysed by means of the Two sample t-test with bonferroni correction for the number of comparisons ($\alpha/3$, $p<0.017$)

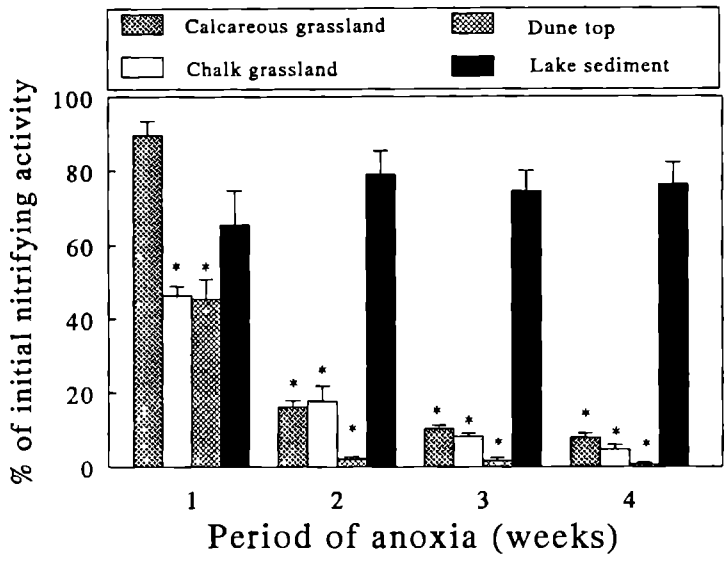

Figure 5 Effect of prolonged anoxic incubation on ammonium-oxidising activities in sediment/soil cores from the root zone of *G maxima* (Drontermeer), a calcareous grassland, a chalkgrassland and a dune top, sampled in May 1995 Initial nitrifying activities were 5 56 (nmol $NO_2^- + NO_3^-$ g^{-1} dry sediment/soil h^{-1}), 46 16, 44 54 and 17 60 for the lake sediment, the calcareous grassland, the chalk grassland and the dune top, respectively Bars represent means ($\pm$1SE) of 5 replicate cores Asterisks indicate significant effects of the anoxic treatments on ammonium-oxidising activities (Tukey's test, $n=5$, $p<0.05$) Except for the lake sediment, homogeneity of variances was achieved after logarithmic transformation of the data

Effect of anoxia

Subjecting the ammonia-oxidising communities to anoxia for a period of 4 weeks, resulted in a drastic decrease in the ammonium-oxidising potential, as compared to the initial capacity; 92% in the calcareous grassland, 95% in the chalk grassland and 99% at the dune top location (Fig.5). Loss of nitrifying capacity already occured after 1 week of anoxic incubation. The ammonium-oxidising potential of the lake sediment was however not affected at all by the anoxic incubation. Subsequent to an anoxic incubation for 4 weeks, the ammonia-oxidising community from the lake sediment resumed nitrification within one hour whereas the ammonia-oxidising communities of the other locations required a substantial lag period (Table 6). The dune top community tended to reveal the most extreme sensitivity to anoxia both in initial capacity and length of lag time required for reactivation.

Table 6 Lag times (hours) of the ammonia-oxidising community after anoxic incubation

Sediment/soil type	Weeks of anoxic incubation			
	1	2	3	4
Calcareous grassland	1 34a	2 39ab	2 46ac	2 20a
Chalk grassland	1 94a	3 26b	3.40a	4 00a
Dune top	2 32a	4 24b	6 15b	7 00c
Lake sediment (*root zone*)	1 56a	1 33a	0 87c	0 66d

Lag time values are calculated as the x-axis intercepts of the linear regression equation of nitrifying activity plots versus time Presented values are means of 5 replicate sediment cores/soil samples Different letters, per week of incubation, indicate significant differences between the different locations (Tukey's test, $p < 0\ 05$)

Discussion

Importance of G. maxima for nitrification and denitrification

The emergent macrophyte, *G. maxima*, had a strong stimulating effect on the numbers of nitrifying bacteria in the Lake Drontermeer sediment in the spring and early summer. The ammonia- and nitrite-oxidising numbers in the root zone were 75 and 50 times as high, respectively, as the numbers found in the bare sediment. The root zone/bare sediment ratio for the ammonium-oxidising activities reached values up to 9. These observations are in agreement with the only other known

report concerning numbers of nitrifiers associated with *G maxima* roots (Both *et al* , 1992a) This other study, however, only sampled the visibly oxidised layer around the roots, whereas the effects we obtained are for complete sediment cores within the vegetation, taking a substantial sediment volume into account which will not directly be influenced by the plant, rendering an even larger overall effect Hansen and Ostergaard-Andersen (1981) reported spring ammonium oxidation rates 3 times as high in the root zone of *Phragmites australis* as compared to the bare sediment These activities, which are within the range we report, are probably over-estimations as the authors measured nitrate and nitrite production during a 24 hour period, which does not exclude growth of nitrifiers Wittgren (1988) found ammonium oxidation rate values, in lysimeters planted with *G maxima* and intermittently supplied with waste water, that were 4 times higher than the highest rates in our study However, the ammonium oxidation rates were lower in the planted plots than in the bare plots, which was explained by ammonium limitation due to plant uptake

The redox potentials in the root zone indicate oxygen release by the roots which stimulates oxidation processes yielding a stabilisation of the redox level The elevation of numbers and activities of the nitrifiers in this study must be due to this oxygen leakage from the roots Stimulation of growth and activity of the nitrifying bacteria as the result of higher ammonium availabilities is not very likely, as relatively little nitrification occurs in the bare sediment despite the higher ammonium availability The decline in number and activity of nitrifiers during the growing season is probably the result of oxygen limitation Oxygen shortage is most likely due to a decrease in the oxygen leakage from the roots as the plants age (Caffrey and Kemp, 1991, Moorehead and Reddy, 1988, Armstrong and Arnstrong, 1988) in combination with elevated heterotrophic microbial oxygen consumption due to the high summer temperatures Ammonium limitation due to plant uptake and microbial immobilisation cannot be totally ruled out within the scope of this study Hence a combination of an increasing oxygen and or nitrogen demand in the sediment in concert with a decrease in the amount of oxygen released by the plant may have resulted in inhibition of the nitrifiers over the course of the growing season Nevertheless, a substantial stimulation of the nitrifying community during a part of the year by *G maxima* could be of critical importance for survival during the following period of the year when no plants and no oxygen are present

Most studies on the impact of oxygen releasing plants on nitrification and denitrification have focused on nitrogen fluxes They have measured the presence of nitrate in the root zone (Cristensen and Sϕrensen, 1986) or the accumulation of nitrogen gas and nitrous oxide after ammonium application (Smith and Delaune, 1984, Reddy *et al* , 1989b) Nitrate reductase levels in plants were also used as indicator for nitrification (Blacquière, 1986, Uhel *et al* , 1989) However, in this study the emphasis was on the dynamics of the community of nitrifying bacteria

during subsequent growing seasons. The dynamic pattern of nitrifier numbers throughout the year would imply high growth- and death rates neither of which are characteristic of nitrifiers. High mortality rate is certainly not likely regarding the presence and activity of the nitrifiers in the oxygen-limited bare sediment during the year, as demonstrated in our study, and long term survival in anoxic sediment layers demonstrated in other studies (Smorzewski and Schmidt, 1991; Jensen *et al.*, 1993). Therefore, the elevated numbers and activities in spring in the root zone probably reflects a high proportion of active or non-resting cells, due to the favourable conditions, which grow in MPN media and are active in short term assays. The significant correlation found for numbers and activities (Table 4), which has rarely been reported, suggests that a large proportion of the counted bacteria can also rapidly be activated in short term activity assays.
It is evident that emergent macrophytes can provide the temporarily conditions, namely oxygen availability, to support the generation of energy by an "activated" nitrifying community.
The activation of the nitrifying community appears to be mirrored by the *in situ* production of nitrite and nitrate, as evident from the high correlation between numbers of ammonia- and nitrite-oxidising bacteria (Table 4), the latter deriving nitrite from the ammonia oxidisers. Interestingly, nitrite-oxidising bacteria have other means of energy generation, which may explain why they outnumbered the ammonia oxidisers to a substantial degree, as previously discussed by Woldendorp and Laanbroek (1989).
The high correlations between the denitrifying activities and the nitrification parameters (Table 4) provide more definite evidence for *in situ* nitrification. The use of chloramphenicol in the denitrification assays prevents *de novo* enzyme synthesis. The necessity of nitrate and nitrite for the induction of the denitrification enzymes and the high correlation between nitrification and denitrification activity implies *in situ* presence of nitrate or nitrite. The strong relation between nitrification and denitrification was not observed however in the spring of 1995. Denitrifying enzyme activity in the root zone was equal to that found in the bare sediment. The higher root biomass in 1995 may have lead to a nitrate limitation for the denitrifying bacteria, explaining the inconsistency with the 1994 results.

Oxygen kinetics and adaptive responses of the ammonia-oxidising community

It is a commonly stated hypothesis that nitrifying bacteria are poor competitors for oxygen compared to heterotrophic bacteria due to their low oxygen affinities (Sharma and Ahlert, 1977; Laanbroek and Woldendorp, 1995). However, most published data on oxygen kinetics of nitrifiers to date have been determined using pure cultures grown in continuous or batch fermenters, measuring oxygen depletion by means of oxygen specific electrodes. We present here the first determination of oxygen kinetics of ammonium oxidation in sediment, taken directly from the field,

using the stochiometrical relation between nitrite/nitrate production and oxygen consumption, thereby excluding heterotrophic respiration The affinity constants for oxygen (K_m) we found for the ammonia- oxidising community in the lake sediment varied between 5 15 μM O_2, which agrees very well with values found in pure culture studies (Laanbroek and Gerards, 1993, Laanbroek *et al* , 1994) Although these values permit nitrification activity at low oxygen concentrations, they are still higher than the range of K_m values (0 02-3μM) reported for heterotrophic bacteria (Sharma and Ahlert, 1977) This difference in half saturation constant for oxygen between nitrifiers and heterotrophs might explain the repression of nitrification during the growing season However, when comparing competitive abilities, the maximum consumption capacity at low substrate concentrations must be considered The specific affinity, defined as V_{max}/K_m which is equivalent to the substrate-sequestering abilities at low substrate concentrations (Healy, 1980), of the ammonia-oxidising community in the lake sediment reveal that the spring 1994 and 1995 communities have greater competitive capabilities for oxygen (Table 3) These specific affinity values can only be compared to heterotrophic bacteria by converting them to oxygen conversion rates per cell For May 1995, we can calculate a specific affinity of 169 nl cell^{-1} h^{-1} which is 7 times the value found for *Escherichia coli* (Button, 1985) and 57 times the values found for pure cultures of *Nitrosomonas europaea* (Laanbroek and Gerards, 1993) Assuming the MPN counts to be representative for the number of active cells present in the nitrification assays, our results indicate that competitive abilities of the ammonia oxidisers on a per cell basis are better than that of heterotrophs This was also demonstrated by comparing oxygen kinetics of pure cultures of the heterotroph *Pseudomonas chlororaphis* and *N europaea*, which were determinded by using exactly the same methods and growth conditions (Bodelier and Laanbroek, 1997) However, taking into account the extreme low yields and growth rates of nitrifiers (Prosser, 1989) it is very likely that heterotrophic organisms will outnumber nitrifiers when oxygen is limiting and sufficient carbon is available This would suggest that the stimulation of nitrification was due to sufficient oxygen release by the roots, making both heterotrophic and nitrification activity possible

There is a large part of the year when the plants are not supplying oxygen, and during these periods the nitrifiers will be confronted with anoxic- or low oxygen conditions, making adaptive traits to these conditions very useful Comparing the ammonia-oxidising community of the lake sediment to communities from permanently oxic dune sand reveals that the ammonia-oxidising comunity in the lake sediment had a higher affinity for oxygen (Table 5) This is also the case with the ammonia oxidising communities of two soils where periodically anoxia and anoxic microsites are present Apparently, the exposure to anoxia leads to higher oxygen affinity The specific affinity of the dune community is equal to that found for the lake sediment This enables them to reach similar conversion rates at low oxygen concentrations on a soil weight basis Specific affinities calculated per cell

are also lower for the dune community (24 nl cell $^{-1}$ h $^{-1}$) as compared to the ammonia-oxidising community in the lake sediment (169 nl cell $^{-1}$ h $^{-1}$) Hence, on a per cell basis the ammonia-oxidising bacteria from the dune sand, which never have to deal with anoxia, will not be very active in sub-oxic environments
Apart from functioning at sub oxic conditions, successful survival in anoxic periods might be a very valuable trait in environments that are periodically oxic/anoxic The ammonia-oxidising community out of the root zone of *G maxima* was not significantly affected by anoxic incubation up to 4 weeks The nitrifying capacity was maintained, whereas the ammonia-oxidising communities from dune sand, a calcareous grassland and a chalk grassland displayed nearly a complete loss of their initial nitrifying capacity and showed substantially longer lag times after 4 weeks of anoxic incubation This survival of long term anoxia of nitrifying bacteria has previously been demonstrated by several studies (Hall and Jeffries, 1984, Smorzewski and Schmidt, 1991, Jensen *et al* , 1993) The ammonia-oxidising community of the lake sediment possesses the ability to react immediately upon the introduction of oxygen at full available capacity, as was also demonstrated with micro electrode studies (Jensen *et al* , 1993) This ability might be very important in reacting upon oxygen released by new roots in the beginning of the growing season, after a period of anoxia of 4-5 months when the shoots of the plants have been absent Moreover, the scavenging of oxygen during the daily fluctuations in oxic/anoxic conditions in the root zone of oxygen-releasing plants, might also be facilitated by this trait
The root zone of emergent macrophytes is an environment with many oxic/anoxic fluctuations, and the adaptations which we have demonstrated may be of prime importance for nitrifier survival in such habitats Whether these adaptations are due to physiological plasticity or the presence of genera or species specialised for nitrification at sub-oxic conditions or survival in anoxic habitats can now also be addressed with the advent of new molecular detection techniques for these organisms

Chapter 6

Community analysis of ammonia-oxidising bacteria, in relation to oxygen-availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation

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Community analysis of ammonia-oxidising bacteria, in relation to oxygen-availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation

Abstract

The rhizosphere of oxygen-releasing roots provides a niche for oxygen-consuming microorganisms such as autotrophic ammonia-oxidising bacteria Nitrifying bacteria inhabiting this plant-affected habitat show adaptations to this periodically anoxic environment in terms of their affinity for oxygen and ability to survive periods of anoxia and their immediate response to the appearance of oxygen The recent application of 16s rDNA-based molecular technologies has allowed for the investigation of ammonia-oxidising bacteria in various environments without the requisite need to culture target organisms In this study the techniques of specific amplification of ammonia oxidiser 16S rDNA fragments by PCR, separation of mixed PCR samples by DGGE (Denaturing Gradient Gel Electrophoresis), and band identification by specific hybridisation with oligonucleotide probes were combined to allow for the comparison of the community composition of multiple samples over space and time DGGE bands of interest were also excised for DNA isolation, reamplification, sequence determination and phylogenetic analysis We compared monthly samples from both the root zone and the bare sediment of a shallow lake inhabited by the emergent macrophyte *Glyceria maxima* to determine the seasonal effects that the plant roots and the oxygen availability might have on the ß-subgroup ammonia-oxidiser populations present Similarly, five different locations in the Netherlands, varing in oxygen availability, were compared Although the presence of two previously defined *Nitrosospira* sequence clusters could be differentially detected in the samples examined, there was no evidence for a particular group which was specific to periodically anoxic environments

Introduction

Plants rooting in flooded soils and sediments contain aerenchymatous tissue in their shoots and roots. By means of this airspace continuum they facilitate the movement of atmospheric or photosynthetic oxygen to the roots (Justin and Armstrong, 1987; Armstrong *et al.*, 1994, Blom and Voesenek, 1996). Thus, root respiration continues, and the accumulation of reduced phytotoxic compounds (i.e Fe^{2+}, Mn^{2+}, S^{2}) is prevented by radial oxygen loss into the surrounding soil or sediment. The plant-released oxygen can be used by a vast number of chemical as well as biological processes One of these processes is the conversion of ammonia into nitrate by nitrifying bacteria. Since this process cannot proceed in the absence of oxygen, oxygen-releasing plants can have a major impact on nitrogen cycling in otherwise anoxic soil or sediment layers The plant-induced nitrate production can subsequently lead to enhanced denitrification, thus elevating nitrogen loss from the ecosystem

A stimulation of nitrification by oxygen-releasing plants has been demonstrated indirectly by MPN counts (Both *et al* , 1992a, Bodelier *et al* , 1996), activity measurements (Hansen and Ostergaard-Andersen, 1981; Engelaar *et al.*, 1995; Bodelier *et al.*, 1996) and evolution of $^{15}N_2$ following addition of $^{15}NH_4^+$ to

microcosms planted with rice (Reddy *et al.*, 1989b). The study of Bodelier *et al.* (1996), showed higher numbers and potential activities of both ammonia- and nitrite-oxidising bacteria in the root zone of the emergent macrophyte *Glyceria maxima* (Hartm.) Holmb. at a freshwater lake location in the Netherlands. This stimulation only occurred in spring and early summer and disappeared later in the growing season. Furthermore, comparison of the lake sediment ammonia-oxidisers with those from a permanently oxic dune soil and two predominantly oxic grassland soils revealed possible adaptations of ammonia-oxidising bacteria to sub-oxic or anoxic habitats.

The seasonal dynamics and adaptations of the ammonia-oxidisers might be reflected by shifts in community composition in concert with the presence or absence of key regulatory substrates such as oxygen and ammonia. However, pure culture isolation would be an imperative step in addressing directly the physiological differences between nitrifying bacteria occurring under different environmental conditions. Unfortunately, this task has proven extremely difficult and tedious for this group of microorganisms, and present culture techniques seem to isolate specifically only a small fraction of the total diversity within the autotrophic ammonia-oxidising bacteria (Prosser, 1989; McCaig *et al.*, 1994; Stephen *et al.*, 1996).

The recent application of molecular biological techniques targeting the 16S rRNA molecule to microbial ecology has provided a viable addition to extant culture-based methods for studying the diversity, composition, and dynamics of microbial communities (Giovanonni *et al.*, 1990; Ward *et al.*, 1990; Amann *et al.*, 1995). The ß-subgroup ammonia-oxidising bacteria represent ideal candidates for analysis via a 16S rDNA-based approach as they comprise a monophyletic group based upon 16S rDNA sequence comparisons, and their laboratory cultivation has proven difficult, time-consuming, and biased for the strains most amiable to the enrichment conditions used (Prosser, 1989; Head *et al.*, 1993; Teske *et al.*, 1994). A number of recent studies have exploited various 16S rDNA-based strategies to study nitrifiers in several natural habitats (McCaig *et al.*, 1994; Hiorns *et al.*, 1995; Voytek and Ward, 1995; Stephen *et al.*, 1996; Kowalchuk *et al.*, 1997) as well as in sewage treatment plants (Wagner *et al.*, 1995; Wagner *et al.*, 1996; Mobarry *et al.*, 1996) and fish culture aquaria (Hovanec and Delong, 1996). Stephen *et al.* (1996) revealed that the ß-subgroup ammonia-oxidising bacteria are comprised of at least seven phylogenetically distinguishable sequence clusters within the *Nitrosomonas* and *Nitrosospira* genera (the latter encompassing the previous genus designations *Nitrosolobus* and *Nitrosovibrio*; Head *et al.*, 1993) (Figure 1). Some novel phylogenetic clusters affiliated with the ammonia-oxidising ß-proteobacteria revealed by Stephen *et al.* (1996) are designated as *Nitrosomonas*- or *Nitrosospira*-like, as there exist as yet no pure culture representatives known to show affinity with them. The molecular survey conducted by Stephen *et al.* (1996) was successful in revealing novel sequence clusters related to the ß-subgroup ammonia oxidisers and provided a database for the engineering of more specific PCR

primers and oligonucleotide probes (Kowalchuk *et al* , 1997, Stephen *et al* , in preparation), but its strategy of cloning and sequencing of PCR-amplified 16S rDNA fragments is very time-consuming and costly and therefore not amiable to the analysis of multiple samples

DGGE (Denaturing Gradient Gel Electrophoresis) offers an alternative to cloning-based approaches by separating the constituent sequences of complex mixtures of PCR products based upon their differential mobility in a gradient of denaturing chemicals (Myers *et al* , 1987) This technique has been adapted for microbial ecological studies by Muyzer *et al* (1993) and has been applied to study the ß-subgroup ammonia-oxidising communities of coastal sand dunes by Kowalchuk *et al* (1997) These authors demonstrated, however, that DGGE pattern alone was not sufficient in determining sequence cluster affinity or demonstrating the identity of co-migrating fragments As with other DGGE-based analyses (Muyzer *et al* , 1995a, Ferris *et al* , 1996, Teske *et al* , 1996), it proved necessary to excise DGGE bands for subsequent DNA isolation, reamplification by PCR, and sequence analysis It is however possible to interpret DGGE patterns more rapidly by the use of oligonucleotide probes targeting signature sequences contained within PCR products derived from organisms of interest (Muyzer *et al* , 1995a, Teske *et al* , 1996) For ß-subgroup ammonia oxidisers, a hierarchical set of hybridisation probes has now been designed and used in conjunction with PCR and DGGE for the identification of the seven described sequence clusters within this group (Stephen *et al* , 1997)

Previous molecular studies of ß-subgroup ammonia oxidisers have shown that specific sequence clusters are associated with certain environments and that shifts in environmental factors such as pH and ammonia concentration can affect the sequence cluster composition of the ammonia-oxidising community (McCaig *et al* , 1994, Stephen *et al* , 1996, Kowalchuk *et al* , 1997) The aim of this study was to investigate whether the seasonal dynamics and adaptations of the ammonia-oxidising community in the root zone of *G maxima*, as was found by Bodelier *et al* (1996), are reflected in the sequence cluster composition of these communities Therefore, the same sediment and soils samples were analysed using the PCR, DGGE and hybridisation approached outlined above Where hybridisation results failed to fully resolve DGGE patterns or where DGGE patterns and hybridisation results were in apparent conflict, DGGE bands were excised for subsequent sequence determination and phylogenetic analysis

Materials and methods

Sampling of sediment and soil

Sediment samples were collected inside and outside the root zone of the emergent macrophyte *Glyceria maxima (Hartm) Holmb* (reed sweet grass) at Lake Drontermeer, near the township of Elburg (52°58'N, 5°50'E) (the Netherlands), as described by Bodelier *et al* (1996), in the period from May 1994 through May 1995 The sediment samples from the root zone of *G maxima* collected in May 1995 were compared to soil samples from three locations differing in oxicity profiles Soil samples were collected from the top of a coastal dune (Meijendel, near The Hague, the Netherlands) (52°08'N, 4°19'E) (permanently oxic), from a chalk grassland in The Gerendal (50°5'N, 5°54'E) in the south of the Netherlands (partially anoxic) and from a calcareous grassland situated in a river levee near the village of Brummen (the Netherlands) (52°5'N, 6°9'E) ("periodically anoxic" by flooding) as described by Bodelier *et al* (1996) Details with respect to the characteristics of the sampled locations are reported by Bodelier *et al* (1996)

DNA isolation

DNA from sediment and soil samples was isolated by a modified protocol of Stephen *et al* (1996) Sediment and soil samples (0 5 g wet weight), 0 5 ml extraction buffer (120 mM K_2HPO_4 [pH 8], 5% CTAB, hexadecyltrimethylammoniumbromide, Sigma), 0 5 g glass beads (0 1 mm, BioSpec Products, Technolab, Alkmaar, the Netherlands) and 0 5 ml phenol/chloroform/isoamylalcohol (25 24 1 v/v, Sigma) were mixed in a 2 ml destruction tube (Anthos Labtec, Heerhugowaard, the Netherlands)
Samples were shaken three times at 5,000 rpm for 30 sec in a mini bead beater (BioSpec) The tubes were cooled on ice between shaking periods After centrifugation (5 min , 3,000 x *g*), 300 μl of the aqueous phase was removed and the rest of the tube's contents re-extracted with an additional 300 μl extraction buffer The two resulting aqueous phases were pooled and twice extracted with 1 vol of chloroform / isoamylalcohol, 24/1, v/v) After centrifugation the DNA was precipitated for at least one hour at -20°C with 0 1 Vol 3*M* CH_3COONa [pH 5 2] and 1 vol isopropanol Centrifugation (15 min , 14,000 x *g*) resulted in a pellet which was subsequently washed with 70% ice-cold ethanol Pellets were allowed to air dry, and DNA was resuspended in 40 μl TE buffer (10 *mM* TRIS, 0 1 *mM* EDTA [pH 8 5], 10 μl loading dye To remove humic compounds, DNA was loaded on a 1% agarose 2% polyvinylpoly-pyrrolidone (PVPP) combination gel as described by Kowalchuk *et al* (1997) and run at 100V for 2 hr
DNA longer than 10 kb was excised and isolated from agarose using QIAquick gel extraction kit (Qiagen Inc , Chatsworth, Ca), DNA was eluted with 50 μl PCR-TE

buffer (10 *mM* TRIS, 0 1 *mM* EDTA [pH 8 5])

PCR conditions

PCR was first performed using between 10 and 100 ng template DNA with the Eubacterial primers pA and pH (Edwards *et al* , 1989) using Tbr polymerase ("Dynazyme", Finnzymes, Iploo, Finland) according to manufacturers recommendations with the following thermocycling program 1 X (2 min 94°C), 30 X (30 sec 94°C, 60 sec 55°C, and 75 sec + 1 sec /cycle 72°C), and 1 X (5 min 72°C) with a reaction volume of 25 μl PCR products (all 25 μl) were examined by electrophoresis in a 0 5 X TBE 1 % low melting point agarose gel (LM-MP agarose, Boehringer, Mannheim, Germany) followed by ethidium bromide staining For all samples, the product of the expected size (1 5 kb) was excised from the gel (total of 100 mg gel material) The gel fragment was melted by heating for 5 min at 65°C, and 1 μl was used as template in a second PCR using the CTO primers (CTOf189-GC, CTO654r) previously described to amplify specifically a 465 bp fragment of the 16S rRNA gene of ammonia-oxidising bacteria belonging to the ß-Proteobacteria (Kowalchuk *et al* , 1997) with the addition of a 5' GC-clamp (Sheffield *et al* , 1987) These 50 μl reactions were performed using Expand High Fidelity polymerase (Boehringer, Mannheim,) according to the manufacturer's specifications using to the following thermocycling program 1 X (1 min 94°C), 25 X (30 sec 92°C, 60 sec 57°C, and 45 sec + 1 sec,/cycle 72°C), and 1 X (5 min 68°C) PCR amplification from plasmid controls was performed by direct use of the CTO primers using the conditions described above accept that 5 ng of DNA was used as template and reaction volumes were 25 μl All reactions were overlaid with an equal volume of mineral oil (Sigma, molecular biology grade) and run on an OmniGene Thermal Cycler (Hybaid, Teddington, UK) Final PCR products were examined by agarose gel electrophoresis (1 5% agarose, 0 5 x TBE) and stained with ethidium bromide for visualisation upon UV illumination

DGGE, blotting and oligonucleotide hybridisation

PCR products recovered with the CTO-primers were subjected to denaturing gradient gel electrophoresis (DGGE) according to the protocol of Muyzer *et al* (1995b) as adapted for use with ammonia-oxidising bacteria by Kowalchuk *et al* (1997) Polyacrylamide gradient gels (8% polyacrylamide, 37 1 acrylamide bis-acrylamide, 0 5 x TAE, 1 5 x 200 x 200 mm) (1xTAE = 0 04 *M* Tris-base, 0 02 *M* acetic acid, 1 0 *mM* EDTA, pH 7 5) with a 40 50% denaturing gradient were poured bottom to top with the aid of a CBS gradient maker (Del Mar, USA) and an Econo-pump (BioRad), at a speed of 5 ml min^{-1} (40 ml gradient volume), where 100% denaturing conditions were defined as 7*M* urea with 40% formamide A 10

ml 'stacking' gel containing no denaturants was added on top, before polymerisation had occurred Gels were run overnight in 0 5 x TAE buffer at 80V or at 200V for 6 hr and at a constant temperature of 60°C using the D-Gene system (Bio-Rad Laboratories, Hercules, CA) DNA was stained with ethidium bromide for 30 min and destained for an equal amount of time DNA was visualised by UV transillumination Gel images were stored using 'The Imager' system (Ampligene, Illkirch, France)
DNA in the polyacrylamide gels was blotted to Hybond-N^+ Nucleic Acid Transfer Membranes (Amersham, UK), using a Transblot SD (Bio-Rad) according to Muyzer *et al* (1995b) After completion of the transfer, the DNA was denatured (DNA-side down) on Whatman 3MM (Whatman) filter paper soaked with 0 4*M* NaOH, 0 6*M* NaCl and similarly neutralised with 1*M* NaCl, 0 5*M* TRIS [pH 8] Membranes were sealed in plastic and stored at 4°C until further use
All hybridisation analyses conducted were using the oligonucleotide probes (Table 1) and hybridisation conditions described by Stephen *et al* (1997) Two nanomoles of each oligonucleotide probe were labelled by incubation (37°C, 30 min) with T4-Polynucleotide Kinase (10 u, New England Biolabs) and 20 μCi of γ-^{32}P-ATP (3000 Ci mMol^{-1}, Amersham International), according to standard reaction buffer conditions (Sambrook *et al* , 1989) All prehybridisations and hybridisations were performed using "Quickhyb" solution (Stratagene, Inc) in a Hybaid "Mini Hybridisation Oven" Autoradiography was by overnight exposure to Fuji-RX X-ray film at -80 °C Membranes were stripped prior to re-probing by washing twice in a large volume of boiling 0 1 SSC/ 0 1 sodium dodecyl sulphate (SDS), followed by rinsing in distilled water and air-drying No attempts to quantify the intensity of radioactive signals were made during the course of this study

Sequence analysis of bands excised from DGGE bands

Bands which were chosen for sequence analysis, were carefully excised from the DGGE gel with a scalpel Only the center-most 50% of each band was excised in order to avoid the lane edges where smearing was observed DNA extraction, reamplification and DNA sequencing were as described in Kowalchuk *et al* (1997) DNA sequence manipulations were performed using the SeqApp program, version 1 9a169 (Gilbert, 1993), and phylogenetic analyses were implemented through PHYLIP 5 57 (Felsenstein, 1993) Distance matrix analyses used the method of Jukes and Cantor (1969) with a masking function to exclude ambiguous data, and phylogenetic tree construction was by neighbour joining (Saitou, 1987) Phylogenetic analysis was performed for 287 positions which could be unambiguously aligned for all sequences used in the alignment Boot-strapping was conducted with 100 replicates using the program SeqBoot (Felsenstein, 1993) Affiliations of recovered ammonia oxidiser-like sequences are shown in Fig 1 Bootstrap support for the sequence clusters were similar to those found previously

(Stephen *et al.*, 1996). New sequences were also tested for homology to known sequences in the EMBL databank using the FASTA program (Pearson and Lipmann, 1988).
All unique sequences have been deposited in the EMBL database under the accession numbers AJ000271-AJ000280. Bands whose nucleotide sequences were determined have been given labels in Figures 2b and 3b which corresponds with the sequence names beginning with a "B" in Figure 1. The addition of an " * " to a band label (Figs 2b and 3b) indicates a difference of one base pair from the given numbered sequence. These differences have been shown to be introduced by PCR at the ambiguous position of the reverse primer (Kowalchuk *et al.*, 1997), and have not been included in the phylogenetic analysis.

Table 1 Sequences and properties of the oligonucleotide probes used in this study Probe specifities and hybridisation conditions are as described by Stephen *et al* (1997)

Probe name	Td(°C)	Target group	Probe sequence 5'-3'
All_Ammo220r	50	All ß-subgroup ammonia-oxidisers	AGCTAATCAGRCATCGG
Spira Cl_1/2/3/4r	50	All *Nitrosospira*	TTTCGTTCCGGCTGAAAG
Monas Cl_5/6r	54	All *Nitrosomonas*	GTAGGCCSTTACCCYACC
Spira Cl_3r	56	Cluster 3 *Nitrosospira*	AGGTATTAGCCGTGACCG
Spira Cl_4r	50	Cluster 4 *Nitrosospira*	TCACAGTTATTAACCGTG

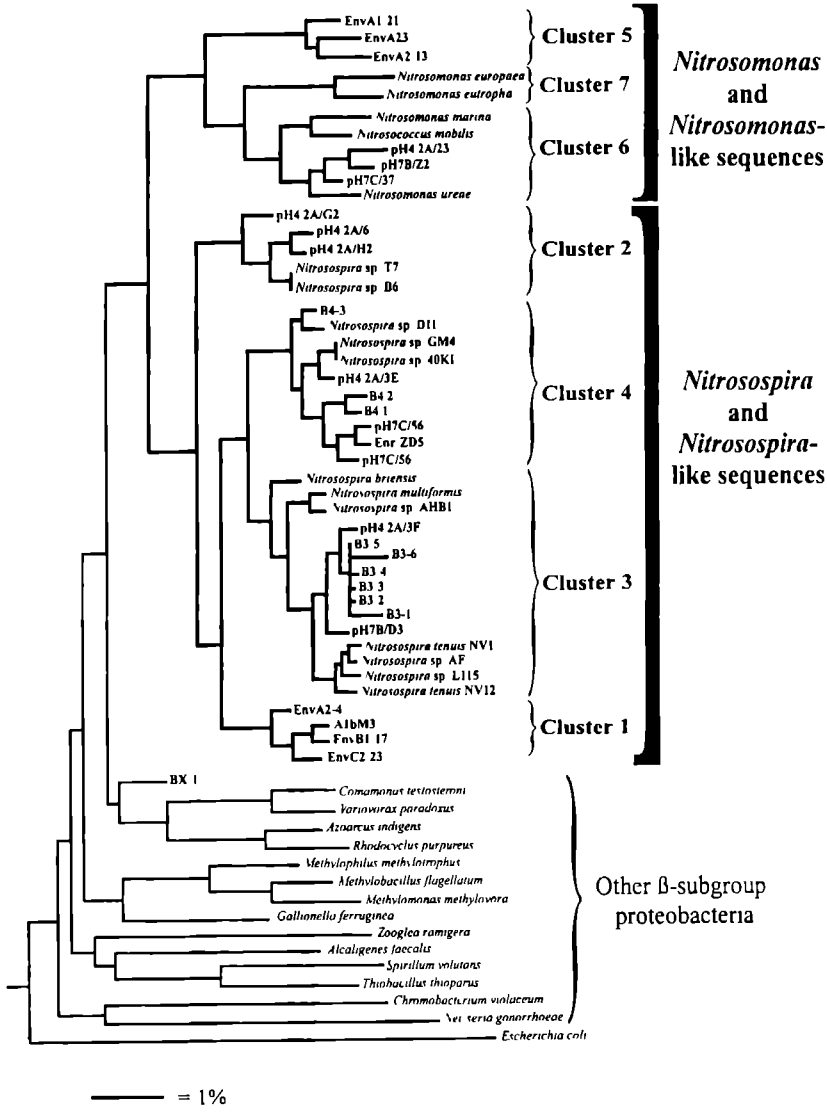

Figure 1 Neighbour-joining tree based upon partial 16S rDNA sequences from β-subgroup ammonia oxidisers Sequence analysis is as described in the text using the cluster designations of Stephen *et al* (1996) Sequences derived from DGGF bands recovered from soil and sediment samples, designated with a "B", correspond to the labelled bands in Figures 2b and 3b Sequence names beginning with "pH" or "Env" refer to clones derived from environmental samples from soil (of the pH indicated) and from marine sediment respectively Sequence A1bM3 is derived from a marine sediment enrichment culture (Stephen *et al* 1996) *Nitrosospira* isolates 40KI, AF, B6, D11, GM4, L115 and T7 were purified from soil (Utaker *et al* , 1996)

Results

Recovery of ammonia-oxidising 16S rDNA fragments from sediment and soil samples

Attempts to recover PCR product from direct amplification with the CTO primer pair, as described in Kowalchuk *et al* (1997) were not consistently successful for all samples, probably due to the inhibitory effect of some copurified humic substances (results not shown) However, after employment of a nested PCR strategy in which DNA extracted from soil is first amplified with Eubacterial primers prior to specific amplification with the CTO primers, ß-subgroup ammonia oxidisers could be detected from all sediment and soil samples analysed For consistency sake, all DGGE and hybridisation analyses were therefore conducted using the nested PCR regime The detection of ß-subgroup ammonia oxidisers is in agreement with previous reports of detectable levels of autotrophic ammonia oxidation for all the sites, and all sampling dates examined (Bodelier *et al* , 1996) Thus, the nested PCR strategy employed was able to detect less than 10^3 culturable cells gram^{-1} dry sediment, as determined previously by MPN analysis (Bodelier *et al* , 1996) This detection sensitivity compares favourably with previous control experiments using soils and cultures seeded with known numbers of nitrite-oxidising bacteria (Degrange and Bardin, 1995)

DGGE and hybridisation analysis of Drontermeer sediment samples

Nested PCR products recovered from the monthly *G maxima* root zone and bare sediment samples from the Lake Drontermeer were subjected to DGGE analysis along with control fragments derived from cloned sequences of known cluster affinity (Figure 2a) (Stephen *et al* , 1996) Fragment mobilities from environmental samples ranged from 44 5 % - 47 2 % denaturant, whereas control ammonia-oxidiser fragment mobilities ranged from 43 0 % - 47 0 % denaturant concentration DGGE patterns from environmental samples were quite simple with usually between two and six detectable bands per sample In both control and environmental DGGE patterns, bands often occurred in doublets, which is consistent with previous results which showed that a single template sequence can give rise to multiple DGGE bands due to an ambiguous position in the CTO reverse primer (Kowalchuk *et al* , 1997) Most samples, excepting the root zone sample from June, 1994 and the bare sediment sample from July, 1994, displayed a clear double band at approximately 45% denaturant Another doublet at approximately 46% denaturant could be observed in many samples although its

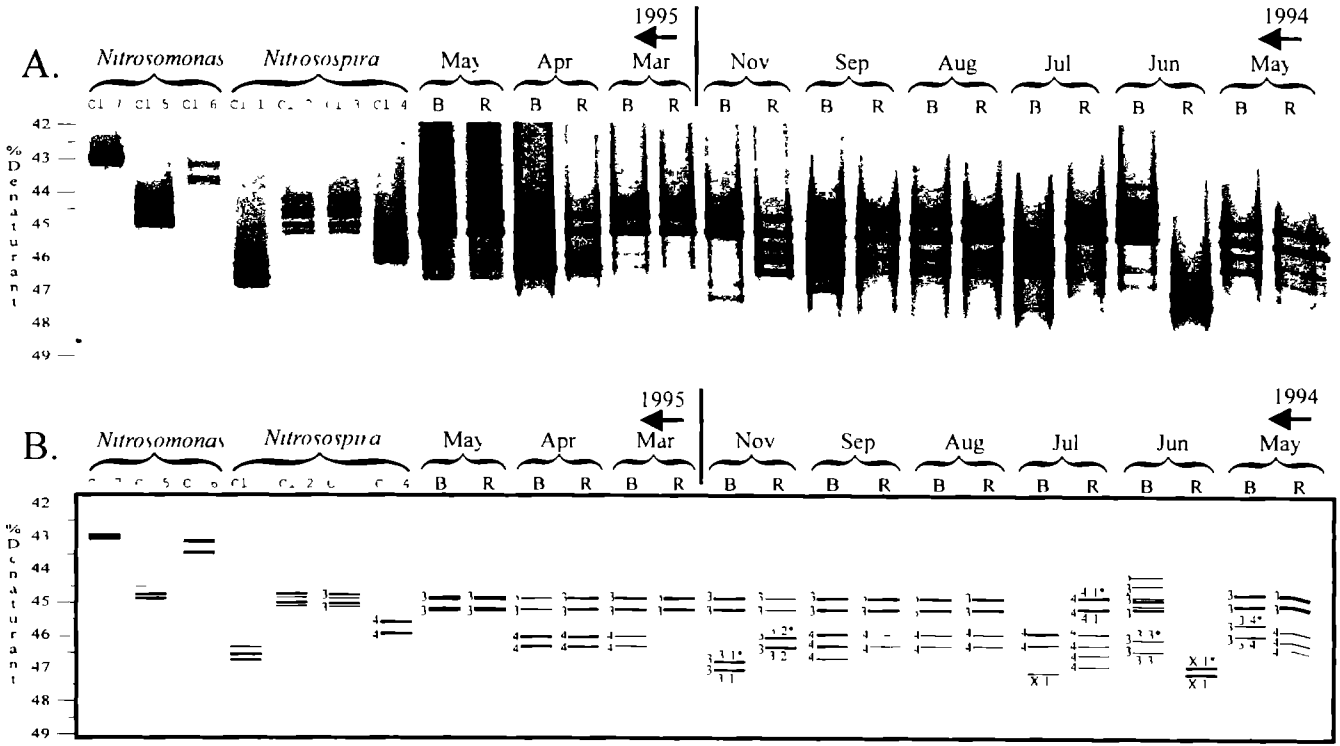

Figure 2 DGGE of Lake Drontermeer monthly samples (a) DGGE analysis of DNA samples extracted from Lake Drontermeer monthly samples following PCR with the CTO primer set Cloned sequences representing the seven previously defined sequence clusters of the ß subgroup ammonia oxidisers are also shown for comparison with environmental samples (Stephen *et al* , 1996) Reference clones were as follows *Nitrosomonas europaea* ATCC 25978, *Nitrosomonas,* cluster 7 EnvA1-21, *Nitrosomonas*-like cluster 5 pH4 2A/23, *Nitrosomonas*, cluster 6, EnvA2-4 *Nitrosospira*-like, cluster 1 pH4 2A/G2, *Nitrosospira*, cluster 2, pH4 2A/3F, *Nitrosospira* cluster 3 pH4 2A/3E, *Nitrosospira* cluster 4 Sediment samples are labelled 'B and 'R' for bare sediment and root zone respectively **(b)** Schematic drawing of DGGE gel from Fig 2a Numbers to the left of drawn bands indicate their sequence cluster affiliations Bands with labels were excised for sequence analysis and their designations correspond those found in Fig 1 The first number in each label indicates the cluster designation and bands having identical labels produced the same nucleotide sequences Sequences which are labelled with an asterisk (*) have a one base pair difference (T for C) with the corresponding clone sequence at position 647 introduced by the ambiguity in the reverse primer (*E coli* numbering Brosius *et al* 1981)

relative intensity was quite variable These two doublets correspond well to the band positions of clusters 2 or 3 and cluster 4 controls, respectively There were no apparent trends in banding patterns with respect to observed seasonal differences in ammonia oxidiser numbers and potential nitrification activities (Bodelier *et al* , 1996) There were also no consistence differences detected between the DGGE patterns from root zone versus bare sediment samples Both types of samples gave similar results in the months of August and September, 1994 and March-May, 1995, whereas other sampling months produced clear differences between the two zones

In order to determine the sequence cluster affinities of the detected DNA fragments, hybridisation analysis was performed using both the genus- and cluster-specific primers described by Stephen *et al* (in preparation) All bands, except for the lower doublet in the June, 1994 root zone sample and the lowest band in the July, 1994 bare sediment sample, showed positive hybridisation with the *Nitrosospira*-specific probe The *Nitrosospira*-negative bands also failed to hybridise with the probe designed to detect all ß-subgroup ammonia oxidisers All *Nitrosospira*-like bands could be further classified by hybridisation analysis into either *Nitrosospira* clusters 3 or 4, and labels to the left of drawn bands in Figure 2b indicate predicted cluster affinity Neither DGGE banding pattern or hybridisation results indicated the recovery of detectable amounts of *Nitrosomonas*-like rDNA sequences

Hybridisation results clearly demonstrated that similar DGGE mobilities were not predictive of common sequence cluster affinities, as was previously demonstrated with known control sequences by Kowalchuk *et al* (1997) For example, the July, 1994 root zone sample contains bands which align well with the control for *Nitrosospira* cluster 3, but hybridisation analysis identified all bands in this sample as belonging to *Nitrosospira* cluster 4 Likewise, the bare sediment samples from June and May, 1994 and the root zone sample from November, 1995 all contain bands whose DGGE migration is similar to that of the *Nitrosospira* cluster 4 control but which are identified as belonging to *Nitrosospira* cluster 3 by hybridisation

DGGE and hybridisation analysis of soil and sediment samples with different oxicity profiles

PCR products recovered from five different soil / sediment types were also examined by DGGE (Figure 3) All samples gave very similar DGGE patterns with all bands within the range of 44 5%-46 5% denaturant The most dominant feature of all DGGE patterns was a doublet at approximately 45% denaturant, although some samples contained other bands lower in the gel All bands reacted positively with the *Nitrosospira*-specific probe, and there were no *Nitrosomonas*-like sequences detected

As with the Drontermeer samples, hybridisation analysis revealed the presence of only *Nitrosospira* clusters 3 and 4 among the ammonia oxidiser sequences detected. Although DGGE banding patterns were quite similar for the five locations examined, the distribution of these two sequence clusters clearly differed, as denoted by the sequence cluster designations given in Figure 3b. This is exemplified by the top doublets for each of the five samples which, although resolving to similar positions after DGGE, give different cluster specific hybridisation results. Both Drontermeer samples (different cores were used as in the analysis of the seasonal dynamics) and the Brummen calcareous grassland soil reveal a predominance of *Nitrosospira* cluster 4 with no detectable signal from other cluster-specific probes. The soil samples from the Gerendal chalk grassland and the Meijendel dune top contained mostly *Nitrosospira* cluster 3, with the latter containing faint bands which hybridised as cluster 4 *Nitrosospira*. These results do not reveal any clear correlation between the presence of a particular sequence cluster and the oxicity profile of the sample.

Sequence analysis of excised bands

In the case where bands could not be classified by hybridisation with cluster-specific probes (June, 1994; root zone sample) and where hybridisation results differed from those predicted by DGGE mobility, DGGE bands were excised for DNA isolation, reamplification and sequence analysis. The bands which were sequenced have been given names in Figures 2b and 3b, and asterisks indicate a one bp difference introduced by the reverse primer during PCR. Identical numbers indicate identical nucleotide sequences and correspond to the numbers assigned the "B" sequences shown in Figure 1. Phylogenetic analysis of the sequence derived from the DGGE doublet at 47.2 % denaturant for the June, 1994 root zone sample revealed that it did not show direct affinity with the monophyletic group formed by all known ß-subgroup ammonia oxidiser 16S rDNA sequences (see Fig.1). This sequence was also detected as a minor band in the July, 1994 bare sediment sample. Although this sequence shows greatest similarity with 16S rDNA sequences from strains and sequences which do fall within the ß-subgroup ammonia oxidiser clade (approximately 92 % identity with a number of sequences), it's phylogenetic position outside this group precludes the assumption that it was derived from an ammonia oxidiser. Based upon the limited phylogenetic information contained within this fragment, it is impossible to determine whether this sequence is derived from a novel group of ammonia-oxidising bacteria, or if it comes from another ß-subdivision Proteobacterium not possessing this trait. Where DGGE mobility and hybridisation analysis were in apparent conflict, DNA sequence analysis confirmed hybridisation results for all cases (compare named bands from Figures 2b and 3b with phylogenetic placement in Figure 1). This result was not unexpected, as Kowalchuk *et al.* (1997) demonstrated overlapping

fragment mobilities between sequence clusters.

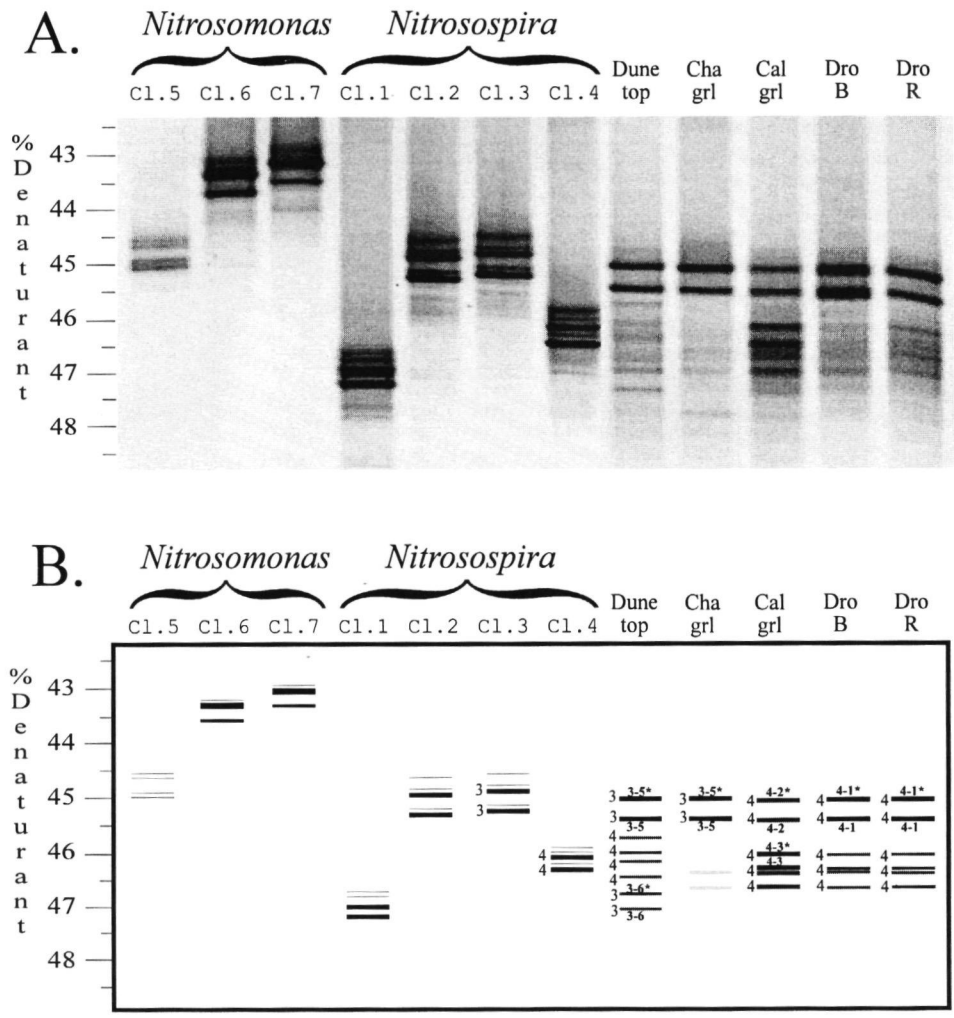

Figure 3: DGGE of soil and sediment samples differing in oxicity.
(a) DGGE analysis of five environmental samples differing in their oxicity profiles, with the same clone references used in Fig 2. Environmental samples are as follows: Dune top = Meijendel; Cha grl = Gerendal; Cal grl = Brummen; Dro B = Lake Drontermeer bare sediment; Dro R = Lake Drontermeer root zone. Band labels are as in Fig 2b. Bands from environmental samples which were too faint for identification have not been labelled.

Discussion

Recovery of ammonia oxidiser 16S rDNA from natural samples

The nested PCR strategy employed was successful in detecting ammonia-oxidiser 16S rDNA from all soil and sediment samples tested MPN analyses showed less than 10^3 culturable ammonia-oxidising bacteria gram^{-1} dry sediment for some samples Detection of this low number of target cells in a background of at least 10^8 non-target cells compares favourably with previous attempts to detect known numbers of inoculated target cells in soils (Picard *et al* , 1992, Degrange and Bardin, 1995) It should be noted that the MPN method detects only cells which are culturable under the selective conditions used, which may underestimate the actual number of target ammonia oxidiser cells present In contrast to Kowalchuk *et al* (1997), a nested PCR strategy was used, as not all samples yielded PCR product after direct amplification with the CTO primers Inhibitory effects of copurified contaminants was observed for some samples, although poor product yield may have also been partially due to the low numbers of target cells in some samples In samples for which both direct and nested PCR results could be compared, DGGE banding patterns were quite similar although there were some differences in relative band intensities (results not shown), as might be caused by only small differences in amplification efficiencies (Suzuki and Giovannoni, 1995) Furthermore, multiple DNA extractions and PCR reactions from the same core yielded reproducible DGGE results (results not shown)

Fidelity of the CTO primers and cluster-specific oligonucleotide probes.

All detected PCR products recovered by the CTO primers during this study were confirmed to have originated from ammonia oxidiser-like organisms by hybridisation, save the double band found in the June, 1994 root zone sample and the lowest band of the July, 1994 bare sediment sample As mentioned above, the phylogenetic placement of the sequence derived from these bands is uncertain, and it can therefore not be assumed to have originated from an ammonia-oxidising strain This is the first example of amplification of a sequence falling outside the ß-subgroup ammonia oxidiser clade by the CTO primer pair from an environmental sample It would be of great interest to characterise this sediment sample further in order to determine if this sequence represents a novel ammonia oxidiser group or a closely related ß-Proteobacterium that possesses the CTO primer binding sites In all cases, nucleotide sequence data confirmed hybridisation results to the sequence cluster level, even when alignment of DGGE bands with known control sequences would have predicted other cluster affinity based upon this criterion In this respect, hybridisation based upon the targeting of cluster-specific signature sequences was far more predictive and reliable than DGGE mobility The sequence

data recovered from DGGE bands also supports the integrity of the *Nitrosospira* sequence clusters proposed by Stephen *et al* (1996, see Fig 1) It should be noted that although all bands appeared to produce a single nucleotide sequence, the possibility that some bands contained a mixture of sequences cannot be discounted, as minor fractions in a template mixture would not be detected by direct sequence analysis

G. maxima root zone versus bare sediment samples, variation in space and time

Only *Nitrosospira* clusters 3 and 4 were detected in Drontermeer sediment samples It is noteworthy that we failed to detect any *Nitrosomonas*-like sequences even though previous results with specific antibodies (Bodelier, unpublished results) did reveal the presence of this genus in the root zone of *G maxima* in Lake Drontermeer Furthermore, *Nitrosomonas*-like sequences, which were most similar to *Nitrosomonas ureae*, could be recovered from MPN cultures from these samples, suggesting that the MPN method selectively enriches for this genus (results not shown) It is unlikely that this lack of *Nitrosomonas* detection is due to failures in the DNA isolation or CTO primer-directed amplification, as the same protocols have revealed the presence of *Nitrosomonas*-like sequences in other soils and sediments even where they are known to be in the minority (Stephen *et al* , in preparation, A Speksnijder and A McCaig, unpublished results)
There were no clear trends corresponding to the observed seasonal fluctuations in ammonia oxidation (Bodelier *et al* , 1996) Although month-to-month differences were apparent in the distribution of *Nitrosospira* sequence clusters 3 and 4, such differences appeared to be random (Fig 2a & b) Furthermore, there were no consistent differences between root zone and bare sediments Although DGGE and hybridisation results revealed clear differences between the root zone and bare sediments for some months, this was not true of all sampling dates and showed no seasonal trend These data do not suggest that the distributions of *Nitrosospira* clusters 3 and 4 are regulated by seasonally varying environmental factors in the root zone of *G maxima* and the nearby bare sediment Thus, the seasonal and locational (root zone vs bare sediment) trends found for nitrification and MPN counts (Bodelier *et al* , 1996) are not mirrored by the ammonia-oxidiser sequence cluster distribution of Lake Drontermeer
Nitrifying bacteria are known to be able to survive long periods of dormancy (Jones and Morita, 1985, Jensen *et al* , 1993) during which cells may show little or no detectable activity and may exhibit a particular aversion to known culture methods This fact may help explain the discrepancy between MPN and molecular results from these samples The 16S rDNA-based techniques used make no distinction between active and dormant cells, whereas MPN analyses may be selective for cells which can be easily activated It would be possible to address this issue by targeting rRNA by specific RT-PCR, followed the same DGGE and

hybridisation analyses (Wagner, 1994, Felske *et al* 1996, Teske *et al* , 1996) The use of functionally relevant structural gene targets, such as the genes coding for ammonia monooxygenase, and studies related to their expression would also be of interest in this respect It is also possible that core to-core variation might confound ammonia oxidiser distribution results Bodelier *et al* (1996) estimated potential ammonium-oxidising activities using five cores per sampling event Although the vernal peak in potential ammonium-oxidising activity was highly significant, some months showed high standard deviations The impact of small-scale spatial heterogeneity might be examined by the analysis of multiple cores from each sampling location and date

Ammonia-oxidising bacteria from sediments and soils differing in oxicity profiles

We did not detect any differences in community structure, as determined by the DGGE and hybridisation analysis, that could be correlated with soil / sediment oxicity for the five different Dutch locations tested (Fig 3a & b) Again, only *Nitrosospira* clusters 3 and 4 were detected, and although clear differences in the distribution of these two clusters were apparent between different locations, such differences did not mirror either the oxygen kinetics of the resident ammonia-oxidising communities or the degree of oxicity of the soil / sediment (Bodelier *et al* , 1996) These results do not support the idea that different sequence clusters of ammonia-oxidising bacteria are specially adapted to survival and growth under oxygen-limited conditions Thus, it may be that the greater affinity for oxygen and the resistance to longer periods of anoxia displayed by sediment communities is an adaptation of a generalist nitrifying community

These results suggests that the differences in nitrifying potential between samples are more likely due to the adaptation of the nitrifying populations, rather than the presence of a phylogenetically definable group specifically well-suited for a given set of environmental conditions The fact that definable sequence clusters exist, and that some of them can be correlated with specific types of environments, suggests that these phylogenetic groupings also reflect physiological differences (Stephen *et al* , 1996, Kowalchuk *et al* , 1997, Stephen *et al* , in preparation) However, it is also possible that very closely related organisms (i e having 16S rDNA sequences which fall within the same cluster) might have highly different physiologies, for instance in their affinity for oxygen This does not seem unlikely considering the physiological and morphological differences displayed by pure cultures within *Nitrosospira* cluster 3 (De Boer *et al* , 1995, Head *et al* , 1993) Of course the possibility remains that other organisms, such as nitrifiers from the λ-subgroup of the Proteobacteria or from other groups which have yet to be described, are responsible for the seasonal fluctuations in potential ammonia-oxidising activities This report shows the use of DGGE of specifically amplified 16S rDNA fragments followed by hybridisation to be a valuable extension of existing techniques for the

biogeographical survey of ammonia-oxidising bacteria of the ß-subgroup of the Proteobacteria in space and time. The use of hybridisation with specific probes proved an essential addition to the DGGE technique for the identification of recovered 16S rDNA fragments, and sequence analysis of excised bands confirmed hybridisation results. The evidence presented demonstrates for the first time that *Nitrosospira*-like organisms may dominate the ammonia-oxidising community in the root zone of oxygen-releasing plants and speaks against the notion that survival and growth in sub-oxic habitats, or after periods of anoxia, is peculiar to certain phylogenetic groupings of ammonia-oxidising bacteria.

Chapter 7

Conclusions and Synthesis

Main conclusions

(1) From pure culture studies it can be concluded that in the root zone of oxygen-releasing plants, availability of ammonia and organic carbon are more important regulating factors in the interactions between nitrifying and denitrifying bacteria than the oxygen concentration

(2) Studies with gnotobiotic microcosms showed the potential of the emergent macrophyte *Glyceria maxima* to stimulate growth of and denitrification by heterotrophic bacteria in its root zone by release of organic compounds

(3) An increase in carbon release, indicated by higher numbers of the denitrifier *Pseudomonas chlororaphis*, was observed with elongation of the day length This observation illustrates that the time in the growing season of the plant must be taken into account when addressing the heterotrophic bacterial community in the root zone

(4) In studies with gnotobiotic microcosms planted with *G maxima*, nitrifying bacteria proved not to be very competitive organisms Activity of *Nitrosomonas europaea* was suppressed by the plant upon elongation of the day length This was due to the increased uptake of ammonium by the plant When sufficient ammonium was present (12 hours photoperiod), *P chlororaphis* suppressed potential nitrifying activities and the growth of *N winogradskyi*, probably by being a better competitor for oxygen than both the nitrifiers, or for nitrite when compared to *N winogradskyi*

(5) In gnotobiotic systems inoculated with the nitrifiers *N europaea* and *N winogradskyi* and the denitrifier *P chlororaphis*, the presence of *G maxima* stimulated potential denitrification activities but did not facilitate the coupling of potential nitrification and denitrification activities

(6) The nitrifying bacteria in the root zone of *G maxima* from a natural ecosystem profited from the oxygen release of the plant only in spring and early summer In the course of the growing season nitrification is suppressed, probably due to ammonium limitation

(7) In the field the presence of *G maxima* concurrently stimulated both denitrifying and, potential nitrification activities The oxic/anoxic interfaces in the sediment, created by the roots, facilitated coupled nitrification-denitrification

(8) Ammonia-oxidising bacteria in the root zone of *G. maxima* appeared to be adapted to their sub-oxic/anoxic habitat. High affinity for oxygen and the immediate and full response to oxygen after a period of anoxia are traits not present in ammonia oxidisers from permanently oxic soils.

(9) The analysis of the ammonia-oxidising community using molecular biological techniques revealed the presence of sequences characteristic for species belonging to the genus *Nitrosospira* inside and outside the root zone of *G. maxima*. *Nitrosomonas*-type sequences were only detected in enrichment cultures.

(10) The seasonal dynamics in numbers and potential activities of the ammonium-oxidising community in the root zone of *G. maxima* were not mirrored by detectable changes in the community composition, determined using the 16S rRNA gene as a marker.

(11) The properties of ammonia oxidisers from the root zone of *G. maxima* in terms of their adaptation to the oxygen profile they encounter could not be related to a specific community composition upon comparison with communities from other locations. This suggests that the observed adaptations are physiological in origin and not the result of the activity of phylogenetically distinct ammonia oxidisers specifically well-suited for sub-oxic/anoxic habitats.

Synthesis

Fluxes of nitrogen compounds at oxic/anoxic interfaces in soils and sediments, created by oxygen-releasing roots, have intensively been studied from agricultural, economical, technical and ecological perspectives In most of these studies the central issue was to assess the amount of nitrogen lost or removed from the ecosystem The extent of nitrogen loss depends primarily on the functioning of the nitrifying and denitrifying bacteria and their mutual interactions with the plant roots and the heterotrophic bacterial community Yet, there is only scarce and incomplete information on the characteristics and functioning of the organisms involved in the nitrogen fluxes in sediments that are oxygenated by roots The aim of this thesis was to help fill this void of knowledge This was done by comparing substrate-sequestering abilities, potential behaviour in model-microcosms and in the field of both nitrifying and denitrifying bacteria

The plant as "niche provider" and "stress factor".

It is evident that the presence of oxygen-releasing roots in flooded soils or sediments can lead to substantial changes in the growth conditions of microbial communities However, the statement often seen in literature that "oxygen-releasing plants stimulate nitrifying and subsequently denitrifying bacteria by supplying the nitrifiers with oxygen" (e g Reddy *et al* , 1989a, Brix, 1994), appears to be an oversimplification, especially with respect to the regulating factors involved
Both experiments with microcosms (Chapter 3 and 4) and field investigations (Chapter 5) have shown the "dualistic" role of oxygen-releasing plants in the functioning of nitrifying and denitrifying bacteria On the one hand, *Glyceria maxima* provides the heterotrophic denitrifiers with the essential carbon sources for their energy generation and the formation of cell material (Chapter 3) On the other hand, the plant can suppress denitrification by depriving denitrifying bacteria of nitrate This idea was supported by microcosm experiments as well as by investigations in the field (Chapter 3 and 5)
The time in the growing season of the plant is a distinct regulating factor in the carbon availability and the uptake of nitrate Carbon availability and nitrogen uptake are directly linked to the length of the daylight period to which the plant is exposed to (Chapter 3 and 4) Denitrification activity in the root zone over the season was correlated with the ammonium-oxidising activity under field conditions, indicating that nitrate was limiting The production of nitrate was significantly stimulated by the presence of *G maxima* However, this was only the case in the beginning of the growing season Increase in plant biomass with the season's progression leads to higher incorporation of mineral nitrogen leading to suppression of the ammonia-oxidising bacteria A high oxygen consumption by heterotrophic

bacteria in the summer due to elevated carbon input by the plant may also lead to suppression of nitrification The microcosm experiments also suggest (Chapter 4) that ammonium limitation caused by plant uptake is the most likely explanation for the repression of the nitrification during the course of the growing season, possibly in combination with oxygen limitation
Hence, the plant is a "niche provider" by supplying the nitrifiers with oxygen and the denitrifiers with carbon At the same time it can be a "stress factor" by removing substrates (i e ammonia and nitrate) which are essential to both groups of bacteria Apparently, the latter is more important on an annual basis The functioning of the bacterial community in the "niche period" will depend on the characteristics and strategies of the respective organisms

Interactions between nitrifiers and denitrifiers

Denitrification in the root zone of *G maxima*, under natural conditions, depends primarily on the activity of the ammonia-oxidising bacteria (Chapter 5) Ammonia and oxygen availability regulate ammonia oxidation Ammonia-oxidisers will not be very competitive in the acquisition of ammonia and oxygen in the presence of plants or heterotrophic bacteria, as demonstrated by chemostat and microcosm experiments (Chapter 2 and 4) This is due to their chemolitho-autotrophic metabolism which does not allow for rapid growth and large biomass formation Nitrifiers require much greater amounts of electron donor than heterotrophs to achieve the same growth performance This situation is not likely to occur in the root zone However, the high specific oxygen affinities of ammonia oxidisers enable them to be active at low oxygen concentrations These lithotrophs compensate for the higher K_m values as compared to heterotrophs by having a high oxygen-conversion rate per cell This makes them, on a cell basis, equally well equipped to function in low-oxygen environments Hence, under oxygen limiting conditions, nitrification will only be suppressed when higher numbers of oxygen-consuming heterotrophs are present This situation is very likely to occur in the root zone where organic carbon is released and ammonium is removed by plant uptake Consequently, the uptake kinetics for ammonia are perhaps even more important as those for oxygen Moreover, the data on the oxidation kinetics of the electron donors for *Nitrosomonas europaea* and *Pseudomonas chlororaphis*, which were presented in Chapter 2, indicate that oxygen only becomes limiting when sufficient electron donors are present Extrapolation to the root zone of oxygen releasing macrophytes suggests that the availability of ammonia and carbon will primarily regulate the activities and the interactions of nitrifying and heterotrophic denitrifying bacteria In other words, based upon pure culture studies, growth- and activity-supporting "threshold" concentrations of the electron donors are higher than that of oxygen, the electron acceptor, for nitrifiers and heterotrophic bacteria In addition to carbon and oxygen release, nitrogen uptake by the plant and the

potentials of the bacteria to acquire nutrients, physical and spatial aspects are also important to the functioning of nitrifiers and denitrifiers in the root zone of oxygen-releasing plants Diffusion rates for oxygen, ammonia, nitrate and organic carbon compounds can also limit bacterial activities The release of oxygen and carbon may be restricted to particular regions of the root system and depends on age of the root, the plant species and on the physical characteristics of the sediment Hence, the spatial arrangement of the bacteria around the roots and the type of sediment will also be important

"Functioning in the niche...surviving the stress"

Apparently, nitrifying bacteria are able to survive in the root zone of oxygen-releasing plants Nitrification can occur in the beginning of the growing season of *G maxima* (Chapter 5), when oxygen is released by the roots, but nitrogen uptake by the plant and oxygen-consuming activities of heterotrophic bacteria are still at a minimum Furthermore ammonia-oxidising bacteria are able to react immediately with full capacity on the presence of ' suitable conditions" (Chapter 5) Even, nitrifiers from the bare sediment react instantaneously upon the introduction of oxygen Although these bacteria may have suffered from oxygen starvation for an extended period of time, they still have preserved their enzymatic machinery during their state of dormancy At the very moment oxygen or ammonia becomes available they can be used This might give nitrifiers and advantage over other microbes which have used up most cell constituents, like proteins, for maintenance The strategy of "sit wait and be prepared ' is definitely a characteristic of nitrifiers, as has also been reported by other investigations Ammonia-oxidisers, isolated from marine environments, and starved in ammonia free medium for 6 months did not change in size, maintained their ammonia-oxidation activity and lowered their endogenous respiration to undetectable levels after 4 weeks of starvation (Jones and Morita, 1985) However, it is yet not clear how cells are able to stay intact' during long periods of energy deprivation

The different physiological responses of the *G maxima* related ammonia-oxidising community could not be related to a phylogenetically different species composition *Nitrosospira*-like bacteria dominate the root zone of *G maxima*, as well as bare sediments, grasslands and dune soils Hence, the different physiological responses observed are likely the consequence of adaptations at the species level rather than of a selection of different species Most physiological studies have been performed with *Nitrosomonas europaea* because it is the most frequently isolated species after selective enrichment Therefore, very little is known about the reactions of *Nitrosospira* to anoxia or sub-oxic conditions, and with respect to the community composition of nitrifying bacteria in the root zone of oxygen-releasing plants, nothing is known In our studies *Nitrosomonas europaea* was detected in the root zone of *G maxima* by applying specific antibody analysis (Chapter 2) In addition, sequences closely related to *Nitrosomonas urea* (Chapter 6) were detected only in

enrichment cultures, which also suggest that they are more easy to cultivate than species belonging to other ammonia oxidising genera The fact that amplification of DNA isolated from the sediments or soils did not reveal the presence of *Nitrosomonas*-like sequences can only be explained by assuming that the number of *Nitrosospira*-like sequences in the environment is much higher leading to a competitive exclusion of the *Nitrosomonas* sequences during direct amplification of DNA by the polymerase chain reaction (PCR) technique
This thesis contributes especially to the knowledge of the functioning and seasonal dynamics of nitrifying bacteria, and the strategy they have adopted to survive Nitrifiers have all the characteristics of K-selectors, putting their cards on longevity and "vegetative" survival, while waiting for good conditions In accordance with plant ecology, Grime would characterise them as "stress tolerators" (Grime, 1979) How they function during the "good times" depends on the amounts of oxygen released and ammonia consumed by the plant and other microbes, with whom they must compete Spatial and temporal separation of nitrification and heterotrophic activities along the root system may also provide opportunities for nitrifying bacteria to be active

Perspective
Nitrification research is still hampered by a lack of pure cultures and the slow growth rate of strains in pure culture Efforts have to be intensified to obtain relevant strains from the root zone of oxygen-releasing plants Adaptations can best be studied in laboratory experiments, yet this requires the culturing of organisms The use of molecular markers may help in the isolation process Nitrifying cells from enrichments, labelled with fluorescent DNA probes may be separated from other bacteria by flow cytometry However, enrichment conditions should imitate the original habitat Again molecular tools can be used to follow changes in community composition during enrichments experiments *In situ* ammonia, oxygen and carbon consumptions in the root zone have yet to be determined The development of new micro-electrodes and techniques like membrane-inlet mass spectrometry might contribute to such activity measurements The use of probes targeting RNA may also produce insight into *in situ* activities Moreover, molecular-based probes in combination with confocal laser-scanning microscopy will reveal the spatial arrangement of bacterial cells around, on and maybe even in roots of oxygen-releasing plants The nature of the adaptations of bacteria may also be investigated experimentally, for instance by subjecting oxic soils to anoxic or sub-oxic conditions and following the community composition and reactions of the ammonia-oxidisers present A challenge will also be to explain the seemingly infinite maintenance of metabolic potential during anoxia displayed by nitrifiers Such endeavours will certainly be worthwhile since this may represent a general ecological principal which facilitates the survival of bacteria in soils and sediments, which has until now eluded laboratory study

Ahlers B., König W. and Bock E. (1990) Nitrite reductase activity in *Nitrobacter vulgaris* FEMS Microbiol Lett 67 121-126

Amann R., Ludwig W. and Schleifer K.-H. (1995) Phylogenetic identification and *in situ* detection of individual cells without cultivation Microbiol Rev 59 143-169

Ambus P. (1993) Control of denitrification enzyme activity in a streamside soil FEMS Microbiol Ecol 102 225-234

Armstrong W. (1971). Radial oxygen losses from intact rice roots as affected by distance from the apex, respiration and waterlogging Physiol Plant 25 192-197

Armstrong J. and Armstrong W. (1988) *Phragmites australis*- A preliminary study of soil oxidizing sites and internal gas transport pathways New Phytol 108 373-382

Armstrong W., Brändle R. and Jackson M.B. (1994). Mechanisms of flood tolerance in plants Acta Bot Neerl 43 307-358

Bakken L. (1988). Denitrification under different cultivated plants effects of soil moisture tension, nitrate concentration and photosynthetic activity Biol Fert Soils 6 271-278

Barber D.A. and Gunn K.B. (1974) The effect of mechanical forces on the exudation of organic substances by the roots of cereal plants grown under sterile conditions New Phytol 73, 39-45

Bédard C. and Knowles R. (1989) Physiology, biochemistry and specific inhibitors of CH_4, NH_4^+ and CO oxidation by methanotrophs and nitrifiers Microbiol Rev 53 68-84

Belser L.W. (1977). Nitrate reduction to nitrite, a possible source of nitrite for growth of nitrite-oxidizing bacteria Appl Environ Microbiol 34 403-410

Belser L.W. (1979). Population ecology of nitrifying bacteria Ann Rev Microbiol 33 309-333

Bennett R.A. and Lynch J.M. (1981) Bacterial growth and development in the rhizosphere of gnotobiotic cereal plants J Gen Microbiol 125 95-102

Beutler H.O. (1985) *In* Bergmeyer HU (ed) Methods of enzymatic analysis, 3rd edn, vol 4, pp 369-376, Verlag Chemie, Weinheim, Deerfield Beach, Basel

Blacquière T. (1986). Nitrate reduction in the leaves and numbers of nitrifiers in the rhizosphere of *Plantago lanceolata* growing in two contrasting sites Plant Soil 61 43-52

Blom C.W.P.M., Voesenek L.A.C.J., Banga M., Engelaar W.M.H.G., Rijnders J.H.M.G., van de Steeg H.M. and Visser E.J.W. (1994) Physiological ecology of riverside species adaptive responses of plants to submergence Ann Bot 74 253-263

Blom C.W.P.M. and Voesenek L.A.C.J. (1996). Flooding the survival strategies of plants TREE 11 290-295

Bock E. (1976). Growth of *Nitrobacter* in the presence of organic matter 2 Chemoorganotrophic growth of *Nitrobacter agilis* Arch Microbiol 108 305-312

Bock E., Sundermeyer-Klinger H., Stackebrandt E. (1983) New facultative litho-autotrophic ammonia-oxidizing bacteria Arch Microbiol 136 281-284

Bock E., Koops H-P. and Harms H. (1986) Cell biology of nitrifying bacteria In Nitrification Special publications of the society for general microbiology, vol 20 pp 17-39 Ed J I Prosser Irl press, Oxford, UK

Bock E., Wilderer P.A., Freitag A. (1988). Growth of *Nitrobacter* in the absence of dissolved oxygen Wat Res 22 245-250

Bodelier P.L.E, Libochant J.A., Blom C.W.P.M and Laanbroek H.J. (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidising bacteria to low oxygen or anoxic habitats Appl Environ Microbiol 62 4100-4107

Bodelier P.L.E. and Laanbroek H.J. (1997) Oxygen uptake kinetics of *Pseudomonas chlororaphis* grown in glucose- or glutamate-limited continuous cultures Arch Microbiol 167 392 395

Bodelier P.L.E., Wijlhuizen A.G., Blom C.W.P.M. and Laanbroek H.J. (1997) Effects of photoperiod on growth of and denitrification by *Pseudomonas chororaphis* in the root zone of *Glyceria maxima*, studied in a gnotobiotic microcosm Plant Soil 190 91-103

Boer de W., Klein Gunnewiek P.J.A. and Laanbroek H.J. (1995) Ammonium-oxidation at low pH by a chemolithotrophic bacterium belonging to the genus *Nitrosospira* Soil Biol Biochem 27 127-132

Boero G. and Thien S. (1979) Phophatase activity and phosphorus availability in the rhizosphere of corn roots *In* The soil-root interface pp 231-242 Eds J L Harley and R S Russel Academic Press, London

Both G.J. (1990) The ecology of nitrite-oxidizing bacteria in grassland soils PhD thesis, University of Groningen pp 89

Both G.J., Gerards S., Laanbroek H.J. (1992a). The occurrence of chemolitho-autotrophic nitrifiers in water saturated-soils Microb Ecol 23 15-26

Both G.J., Gerards S. and Laanbroek H.J. (1992b). Kinetics of nitrite oxidation in two *Nitrobacter* species grown in nitrite-limited chemostats Arch Microbiol 157 436 441

Brandle R.A. and Crawford R.M.M. (1987) Rhizome anoxia tolerance and habitat specialization in wetland plants In Plant life in aquatic and amphibious habitats pp 397-410 Ed R M M Crawford Blackwell Scientific, Oxford

Breemen van N. and Dijk van H.F.G. (1988) Ecosystems effects of atmospheric deposition of nitrogen in the Netherlands Environ Poll 54 249-274

Bremner J.M. and Blackmer A.M. (1981) Terrestrial nitrification as a source of atmospheric nitrous oxide In Denitrification, nitrification and atmospheric nitrous oxide pp 151-170 Ed C C Delwiche Wiley and Sons Inc, New York

Brix H. (1994). Functions of macrophytes in constructed wetlands Wat Sci Tech 29 71-78

Brix H. and Schierup H.H. (1990) Soil oxygenation in constructed reed beds the role of macrophyte and soil atmosphere interface oxygen transport Adv Wat Poll Contr 11 53-66

Brosius J., Dull T.L., Sleeter D.D. and Noller H.F. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli* J Mol Biol 148 107-127

Brunel B., Janse D., Laanbroek H.J. and Woldendorp J.W. (1992) Effect of transient oxic conditions on the composition of the nitrate-reducing community from the rhizosphere of *Typha angustifolia* Microb Ecol 24 51-56

Button D.K, (1985). Kinetics of nutrient-limited transport and microbial growth Microbiol Rev 49 270-297

Button D.K. (1994) The physical base of marine bacterial ecology Microb Ecol 28 273 285

Caffrey J.M. and Kemp W.M. (1991). Seasonal and spatial patterns of oxygen production, respiration and root-rhizome release in *Potamogeton perfoliatus* L and *Zostera marina* L Aquat Bot 40 109 128

Caffrey J.M. and Kemp W.M. (1992). Influence of the submersed plant, *Potamogeton perfoliatus*, on nitrogen cycling in estuarine sediments Limnol Oceanogr 37 1483-1495

Carlson C.A. and Ingraham J.L. (1983). Comparison of denitrification by *Pseudomonas stutzeri, Pseudomonas aeruginosa and Paracoccus denitrificans* Appl Environ Microbiol 45 1247-1253

Cavari B.Z. (1977) Nitrification potential and factors governing the rate of nitrification in lake Kinneret Oikos 28 285 290

Christensen P.B. and Sørensen J. (1986). Temporal variation of denitrification activity in plant-covered, littoral sediment from lake Hampen, Denmark Appl Environ Microbiol 51 1174-1179

Christensen S. and Tiedje J.M. (1988). Sub-parts per-billion nitrate method use of an N_2O-producing denitrifier to convert NO_3 or $^{15}NO_3$ to N_2O Appl Environ Microbiol 54 1409-1413

Clevering O.A and van der Putten W.H. (1995) Effects of detritus accumulation on the growth of *Scirpus maritimus* under greenhouse conditions Can J Bot 73 852-961

Coyne M.S. and Tiedje J.M. (1990). Induction of denitrifying enzymes in oxygen limited *Achromobacter cycloclastes* continous culture FEMS Microbiol Ecol 73 263-270

Crutzen P.J. (1981). Atmospherical chemical processes of the oxides of nitrogen, including nitrous oxide In Denitrification, nitrification and atmospheric nitrous oxide pp 17-44 Ed C C Delwiche Wiley and sons Inc, New York

Curl A. and Truelove B. (1986) The rhizosphere pp 55-91 Springer Verlag, Berlin

DeGrange V. and Bardin R. (1995) Detection and counting of *Nitrobacter* populations in soil by PCR Appl Environ Microbiol 61 2093-2098

Dendooven L. and Anderson J.M. (1995) Maintenance of denitrification potential in pasture soil following anaerobic events Soil Biol Biochem 27 1251-1260

Downes M.T. (1988). Aquatic nitrogen transformations at low oxygen concentrations Appl Environ Microbiol 54 172-175

Drew M.C. (1983) Plant injury and adaptation to oxygen defeciency in the root enviroment a review Plant Soil 75 179-199

Dykyjova D. (1978) Nutrient uptake by littoral communities of helophytes *In* Ecological studies 28 Pond littoral ecosystems pp 257-277 Eds D Dykyjova and J Kvet Springer Verlag, Berlin

Edwards U., Rogall T., Blöcker H., Emde M. and Böttger E.C. (1989) Isolation and direct complete nucleotide determination of entire genes Characterization of a gene coding for 16S ribosomal RNA Nucl Acids Res 17 7843-7853

Egmond van F. (1978) Nitrogen nutritional aspects of the ionic balance of plants *In* Nitrogen in the environment pp 171-189 Eds D R Nielsen and J G Macdonald Academic press, New York

Eisenthal R. and Cornish-Bowden A. (1974) The direct linear plot, a new graphical procedure for estimating enzyme kinetic parameters Biochem J 139 715-720

Engelaar W.M.H.G., Bodelier P.L.E., Laanbroek H.J. and Blom C.W.P.M. (1991) Nitrification in the rhizosphere of a flooding-resistant and a flooding-non-resistant *Rumex* species under drained and waterlogged conditions FEMS Microbiol Ecol 86 33-42

Engelaar W.M.H.G, van Bruggen M.W., van den Hoek W.P.M., Huyser M.A.H. and Blom C.W.P.M. (1993) Root porosities and radial oxygen losses of *Rumex* and *Plantago* species as influenced by soil pore diameter and soil aeration New Phytol 125 565-574

Engelaar W.M.H.G., Symens J.C., Laanbroek H.J., Blom C.W.P.M. (1995) Preservation of nitrifying capacity and nitrate availability in waterlogged soils by radial oxygen loss from roots of wetland plants Biol Fert Soils

20 243-248

Epp M.A. and Chanton J.P. (1993). Application of the methyl fluoride technique for the determination of rhizopshere methane oxidation J Geophys Res 98 18413-18422

Felsenstein J. (1993) Phylip Phylogeny inference package Seattle, Wash

Felske A., Engelen B., Nubel U. and Backhaus H. (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis Appl Environ Microbiol 62 4162-4167

Ferguson S.J. (1994) Denitrification and its control Antonie van Leeuwenhoek 66 89-110

Ferris M.J., Muyzer G. and Ward D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community Appl Environ Microbiol 62 340-346

Firestone M.K. and Davidson E.A. (1989) Microbiological basis of NO and N_2O production and consumption in soil In Exchange of trace gasses between terrestrial ecosystems and the atmosphere pp 7 21 Eds M O Andreae and D S Schimel Dahlem Workshop reports, John Wiley and Sons, Chichester

Flessa H. and Fischer W.R. (1992) Plant-induced changes in the redox potentials of rice-rhizospheres Plant Soil 143 55-60

Focht D.D. and Verstraete W. (1977). Biochemical ecology of nitrification and denitrification In Advances in Microbial Ecology, vol 1 Plenum Press, New York, London pp 135-214

Freitag A., Rudert M., Bock E. (1987). Growth of *Nitrobacter* by dissimilatoric nitrate reduction FEMS Microbiol Lett 48 105-109

Frenzel P., Rothfuss F. and Conrad R. (1992). Oxygen profiles and methane turnover in a flooded rice microcosm Biol Fert Soils 14 84-89

Gamble T.N., Betlach M.R., Tiedje J.M. (1977) Numerically dominant denitrifying bacteria from world soils Appl Environ Microbiol 33 926-939

Garcia J.L. (1975) La dénitrification dans la rhizospere du riz Soc bot Fr , Coll Rhizosphère 122 131-138

Gerritse J., Schut F. and Gottschal J.C. (1992). Modelling of mixed chemostats cultures of an aerobic bacterium *Comamonas testosteroni*, and a anaerobic bacterium *Veillonella alcalescens* comparison with experimental data Appl Environ Microbiol 58 1466-1476

Gilbert B. and Frenzel P. (1995). Methanotrophic bacteria in the rhizopshere of rice microcosms and their effect on porewater methane concentration and methane emission Biol Fert Soils 20 93-100

Gilbert D.G. (1993) SeqApp sequence alignment editor Bloomington, Indiana Available from author by ftp (ftp bio indiana edu)

Giovannoni S.J., Britschgi T.B., Moyer C.L. and Field K.G. (1990) Genetic diversity in Sargasso Sea bacterioplankton Nature (London) 345 60-63

Goreau T.J., Kaplan W.A., Wofsy S.C., Malroy M.B., Valois F.W., Watson S.W. (1980) Production of NO_2 and N_2O by nitrifying bacteria at reduced concentrations of oxygen Appl Environ Microbiol 40 526 532

Grime J.P. (1979) Plant strategies and vegetation processes John Wiley, Chichester

Grime J.P., Hodgon J.G. and Hunt R. (1988) Comparative plant ecology Unwin and Hyman, London pp 742

Guest J.R. (1995). The Leeuwenhoek lecture 1995 Adaptation to life without oxygen Phil Trans R Soc Lond B 350 189-202

Hall G.H. and Jeffries C. (1984) The contribution of nitrification in the water column and profundal sediments to the total oxygen deficit of the hypolimnion of a mesotrophic lake (Grasmere, English Lake district) Microb Ecol 10 37-46

Hanaki K., Wantawin C. and Ohgaki S. (1990) Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor Wat Res 24 297-302

Hansen J.I. and Ostergaard Andersen F. (1981) Effects of *Phragmites australis* roots and rhizomes on redox potentials, nitrification and bacterial numbers in the sediment *In* A Broberg and T Tiren (ed), 9th Nordic symposium on sediments 1981

Hanson R.S. and Hanson T.E. (1996). Methanotrophic bacteria Microbiol Rev 60 439-471

Harrison D.E.F. and Loveless J.E. (1971) Transient responses of facultatively anaerobic bacteria growing in chemostat culture to a change from anaerobic to aerobic conditions J Gen Microbiol 68 45-52

Head I.M., Hiorns W.D., Embley T.M., McCarthy A.J. and Saunders J.R. (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences J Gen Microbiol 139 1147-1153

Healy F.P. (1980) Slope of the Monod equation as an indicator of advantage in nutrient competition Microb Ecol 5 281 286

Helal H.M. and Sauerbeck D.R. (1983) Methods to study turnover processes in soil layers of different proximity to roots Soil Biol Biochem 15 223-225

Hernandez D. and Rowe J.J. (1988) Oxygen inhibition of nitrate uptake is a general regulatory mechanism in nitrate respiration J Biol Chem 263 7937-7939

Hiorns W.D., Hastings R.C., Head I.M., McCarthy A.J., Saunders J.R., Pickup R.W. and Hall G.H. (1995). Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment Microbiology 141 2793-2800

Hφjberg O. and Sφrensen J. (1993) Microgradients of microbial oxygen consumption in a Barley rhizosphere model system Appl Environ Microbiol 59 431-437

Hovanec T.A. and DeLong E.F. (1996) Comparitive analysis of nitrifying bacteria associated with freshwater and marine aquaria Appl Environ Microbiol 62 2888-2896

Hynes R.K. and Nelson L.M. (1990) Interactions among nitrogen-transforming bacteria and nitrogen fixing *Pisum sativum* L in laboratory sand columns Plant Soil 122 157-167

Iizumi H. and Hattori A. (1980) Nitrate and nitrite in interstitial waters of eelgrass beds in relation to the rhizosphere J Exp Mar Biol Ecol 47 191-201

Janse J.D. (1991) Pathovar discrimination within *Pseudomonas syringae subsp savastanoi* using whole cell fatty acid analysis and pathogenicity as criteria Syst Appl Microbiol 14 79-84

Jensen K., Revsbech N.P. and Nielsen L.P. (1993) Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate Appl Environ Microbiol 59 3287-3296

Jones R.D. and Morita R.Y. (1985) Survival of a marine ammonium oxidizer under energy-source deprivation Mar Ecol Prog Ser 26 175-179

Jukes T.H. and Cantor C.R. (1969) Evolution of protein molecules, p 21-132 *In* H N Munro (ed), Mammalian protein metabolism Academic ress, New York

Justin S.H.F.W. and Armstrong W. (1987) The anatomical characteristics of roots and plant response to soil flooding New Phytol 106 465-495

Kalthoff H., Rehr S., Sundermeyer H., Renwantz L., Bock E. (1979) A comparison by means of antisera and lectins of sugar structures of *Nitrobacter winogradskyi* and *N agilis* Curr Microbiol 2 375-380

Keeney D.R., Sahrawat K.L. (1986). Nitrogen transformations in flooded rice soils Fert Res 9 15-38

Kester R.A., de Boer W. and Laanbroek H.J. (1996) Short exposure to acetylene to distinguish between nitrifier and denitrifier nitrous oxide production in soil and sediment samples FEMS Microbiol Ecol 20 111-120

Kester R.A., Meijer M.E., Libochant J.A., de Boer W. and Laanbroek H.J. The contribution of nitrification and denitrification to the NO and N_2O emission of an acid forest soil, a river sediment and a fertilized grassland soil Soil Biol Biochem In press

Kester R.A., de Boer W. and Laanbroek H.J. The production of NO and N_2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration Submitted

Killham K. (1986). Heterotrophic nitrification In Nitrification pp 117 127 Ed J I Prosser Special publications of the the society for general microbiology, vol 20 IRL Press, Oxford, UK

Klemedtsson L., Berg P., Clarholm M. and Schnurer J. (1987a) Microbial nitrogen transformations in the root environment of barley Soil Biol Biochem 19 551-558

Klemedtsson L., Svensson B.H., Rosswall T. (1987b) Dinitrogen and nitrous oxide produced by denitrification and nitrification in soil with and without barley plants Plant Soil 99 303-319

Kludze H.K., Delaune R.D. and Patrick W.H. Jr. (1993). Aerenchyma formation and methane and oxygen exchange in rice Soil Sci Soc Am J 57 386-391

Kludze H.K. and Delaune R.D. (1996) Soil redox intensity effects on oxygen exchange of Cattail and Sawgrass Soil Sci Soc Am J 60 616-621

Knowles R. (1982). Denitrification Microbiol Rev 46 43-70

Koike B.I. and Hattori A. (1975). Growth of a denitrifying bacterium, *Pseudomonas denitrificans*, under aerobic and denitrifying conditions J Gen Microbiol 88 1-10

Körner H. and Zumft W.G. (1989). Expression of denitrification enzymes in response to dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri* Appl Environ Microbiol 55 1670-1676

Kowalchuk G.A., Stephen J.R., de Boer W., Prosser J.I., Embley T.M. and Woldendorp J.W. (1997) Analysis of ammonia-oxidizing bacteria of the ß subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of pcr-amplified 16S ribosomal DNA fragments Appl Environ Microbiol 63 1489-1497

Krümmel A. and Harms H. (1982) Effect of organic matter on growth and cell yield of ammonia-oxidizing bacteria Arch Microbiol 133 50-54

Kuenen G.J. and Robertson L.A. (1987). Ecology of nitrification and denitrification In The nitrogen and sulphur cycles pp 161-218 Eds J A Cole and S Ferguson Society for general microbiology symposium 42 Cambridge University Press

Laan P., Berrevoets M.J., Lythe S., Armstrong W. and Blom C.W.P.M. (1989a) Root morphology and aerenchyma formation as indicators of the flood-tolerance of Rumex species J Ecol 77 693-703

Laan P., Smolders A., Blom C.W.P.M. and Armstrong W. (1989b) The relative role of internal aeration, radial oxygen losses, iron exclusion and nutrient balance in flood tolerance of Rumex species Acta Bot Neerl 38 131-145

Laanbroek H.J. (1990) Bacterial cycling of minerals that effect plant growth in waterlogged soils a review Aquat Bot 38 109-125

Laanbroek H.J. and Gerards S. (1993). Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in mixed continuous cultures Arch Microbiol 159 453-459

Laanbroek H.J., Bodelier P.L.E. and Gerards S. (1994). Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different oxygen concentrations Arch Microbiol 161 156-162

Laanbroek H.J. and Woldendorp J.W. (1995) Activity of chemolithotrophic nitrifying bacteria under stress in natural soils *In* Advances in Microbial Ecology, volume 14 pp 275-304 Ed J Gwynfryn Jones Plenum Press, New York

Lindau C.W., Delaune R.D., Patrick W.H.jr., Bollich P.K. (1990). Fertilizer effects on dinitrogen, nitrous oxide, and methane emissions from lowland rice Soil Sci Soc Am J 54 1789-1794

Lindau C.W., Delaune R.D. (1991). Dinitrogen and nitrousoxide emission and entrapment in *Spartina alterniflora* saltmarsh soils following addition of N-15 labelled ammonium and nitrate Estuar Coast Shelf S 32 161-172

Longmuir J.S. (1954). Respiration rate of bacteria as a function of oxygen concentration Biochem 57 81-86

Marschner H. and Romheld V. (1996). Root induced changes in the availability of micronutrients in the rhizosphere In Plant roots The hidden half pp 557 579 Eds Y Waisel, A Eshel and U Kafkafi Marcel Dekker Inc , New York

Martens R. (1982) Apparatus to study the quantative relationship between root exudates and microbial populations in the rhizosphere Soil Biol Biochem 14 315-317

McCaig A.E., Embley T.M. and Prosser J.I. (1994) Molecular analysis of enrichment cultures of marine ammonium oxidisers FEMS Microbiol Lett 120 363-368

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria Appl Environ Microbiol 62 2156-2162

Moorehead K.K. and Reddy K.R. (1988) Oxygen transport through selected aquatic macrophytes J Environ Qual 17 138-142

Mosier A.R., Mohanty S.K., Bhadrachalam A., and Chakravorti S.P. (1990) Evolution of dinitrogen and nitrousoxide from the soil to the atmosphere through rice plants Biol Fert Soils 9 61-67

Muyzer G., De Waal E.C. and Uitterlinden A.G. (1993) Profiling complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA Appl Environ Microbiol 59 695-700

Muyzer G., Teske A., Wirsen C.O. and Jannasch H.W. (1995a) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples be denaturing gradient gel electrophoresis of 16S rDNA fragments Arch Microbiol 164 165-174

Muyzer, G., Hottentrager S., Teske A. and Wawer C. (1995b) Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA A new molecular approach to analyze the genetic diversity of mixed microbial communities *In* A D I Akkermans, J D Van Elsas, and F J De Bruijn (eds) Molecular microbial ecology manual Kluwer, Dordrecht, the Netherlands

Myers R.M., Maniatis T. and Lerman L.S. (1987) Detection and localization of singe base changes by denaturing gradient gel electrophoresis Methods Enzymol 155 501-527

Niel van E.W.J. (1991) Nitrification by heterotrophic denitrifiers and its relationship to autotrophic nitrification PhD thesis, TUD, the Netherlands

Nijburg J.W., Coolen M.J.L., Gerards S., Klein Gunnewiek P.J.A. and Laanbroek H.J. (1997) The influence of different nitrate inputs and the presence of *Glyceria maxima* on the composition and activity of the dissimilatory nitrate reducing community Appl Environ Microbiol 63 931-937

Odham Ö., Tunlid A., Valeur A., Sundin P. and White D.C. (1986) Model system for studies of microbial dynamics at exuding surfaces such as the rhizosphere Appl Environ Microbiol 52 191-196

Ohashi A., Viraj de Silva D.G., Mobarry B., Manem J.A., Stahl D.A., Rittmann B.E. (1995) Influence of the substrate C/N ratio on the surface structure of multi-species biofilms consisting of nitrifiers and heterotrophs Wat Sci Tech 32 75-84

Olsen K.R. and Andersen F.O. (1994) Nutrient cycling in shallow, oligotrophic Lake Kvie, Denmark Hydrobiologia 275/276 225-265

Painter H.A. (1986). Nitrification in the treatement of sewage and waste treatement In Nitrification pp 185-211 Ed J I Prosser Special publications of the society for general microbiology, vol 20 IRL Press, oxford, UK

Patureau D., Bernet N. and Moletta R. (1996). Effect of oxygen on denitrification in continuous chemostat culture with *Comamonas sp* SGLY2 J Ind Microbiol 16 124-128

Pearson W.R. and Lipmann D.J. (1988) Improved tools for biological sequence analysis Proc Nat Acad Sci USA 85 2444 2448

Pedersen O. and Sand-Jensen K. (1995) Diel pulses of O_2 and CO_2 in sandy lake sediments inhabited by *Lobelia Dortmanna* Ecology 76 1536-1545

Picard C., Ponsonnet C., Paget E., Nesme X. and Simonet P. (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction Appl Environ Microbiol 58 2717-2722

Ponnamperuma F.N. (1984) Effects of flooding on soils In Flooding and plant growth pp 9-45 Ed T T Kozlowski Academic Press, New York

Poole R.K. (1983) Bacterial cytochrome oxidases A structurally and functionally diverse group of electron-transfer proteins Biochim Biophys Acta 726 205-243

Poth M. and Focht D.D. (1985). ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea* an examination of nitrifier denitrification Appl Environ Microbiol 49 1134-1141

Potvin C. and Roff D.A. (1993) Distribution-free and robust statistical methods viable alternatives to parametric statistics? Ecology 74 1617-1628

Prade K. and Trolldenier G. (1990) Denitrification in the rhizosphere of rice and wheat seedlings as influenced by plant K-status, air filled porosity and substrate organic matter Soil Biol Biochem 22 769-773

Prosser J.I. (1989) Autotrophic nitrification in bacteria Adv Microbial Physiol 30 125-181

Reddy K.R. and Patrick W.H.jr. (1984). Nitrogen transformations in flooded soils and sediments CRC Environ Contr 13 273-309

Reddy K.R. and Patrick W.H.jr. (1986). Fate of fertilizer nitrogen in the rice root zone Soil Sci Soc Am J 50 649-651

Reddy K.R., D'Angelo E.M. and DeBusk T.A. (1989a). Oxygen transport through aquatic macrophytes the Role in wastewater treatment J Environ Qual 19 261-267

Reddy K.R., Patrick W.H.jr. and Lindau C.W. (1989b). Nitrification-denitrification at the plant root-sediment interface in wetlands Limnol Oceanogr 34(6) 1004-1013

Rees ap. T., Jenkins L.E.T., Smith A.M., and Wilson P.M. (1987) The metabolism of flood-tolerant plants *In* Plant life in aquatic and amphibious habitats pp 227-238 Ed R M M Crawford Blackwell Scientific, Oxford

Rehr B. and Klemme J-H. (1989) Competition for nitrate between denitrifying *Pseudomonas stutzeri* and nitrate ammonifying enterobacteria FEMS Microbiol Ecol 62 51-58

Robertson L.A. and Kuenen G.J. (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria Antonie van Leeuwenhoek 57 139-152

Robertson L.A., Dalsgaard T., Revsbech N-P and Kuenen J.G. (1995). Confirmation of 'aerobic' denitrification in batch cultures, using gas chromatography and ^{15}N mass spectrometry FEMS Microbiol Ecol 18 113-120

Roelofs J.G.M. (1983). Impact of acidification and eutrophication on macrophyte communities in soft waters in the Netherlands 1 Field observations Aquat Bot 17 139-155

Saitou N. and Nei M. (1987) The neighbor joining method A new method for reconstructing phylogenetic trees Mol Biol Evol 4 406-425

Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular cloning a laboratory manual 2nd ed Cold Spring Harbor Press, Cold Spring Harbor, NY

Scaglia J., Lensi R. and Chalamet A. (1985) Relationship between photosynthesis and denitrification in planted soil Plant Soil 84 37-43

Schmidt E.L. (1982) Nitrification in soil In Nitrogen in agricultural soils Agronomy monograph nr 22 pp 253-288 Ed F Stevenson American Society of Agronomy, Inc, Madison USA

Schmidt E.L. and Belser L.W. (1994) Autotrophic nitrifying bacteria *In* Methods of soil analyses Part 2,Microbiological and biochemical properties pp 159-177 Eds S H Mickelson and J M Bigham Soil Science Society of America, Inc Madison

Seitzinger S.B. (1994). Linkages between organic matter mineralization and denitrification in eight riparian wetlands Biogeochemistry 25 10 39

Sharma B. and Ahlert R.C. (1977) Nitrification and nitrogen removal Wat Res 11 897-925

Sheffield V.C., Cox D.R., Lerman L.S. and Myers R.M. (1987) Attachment of a 40-base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single base changes Proc Natl Acad Sci USA 86 232-236

Sman van der A.J.M. (1992). Flooding resistance and life histories of short lived floodplain herbs PhD thesis, University of Nijmegen

Smirnoff N. and Crawford R.M.M. (1983) Variation in the structure and response to flooding of root aerenchyma in some wetland plants Ann Bot 51 237-249

Smith C.J. and Delaune R.D. (1984). Effects of rice plants on nitrification and denitrification loss of nitrogen under greenhouse conditions Plant Soil 79 287-290

Smorzewski W.T. and Schmidt E.L. (1991) Numbers, activities and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment Can J Microbiol 37 828-839

Snedecor G.W. and Cochran W.G. (1973) Statistical methods The Iowa State university press, Iowa, USA

Sorrell B.K. and Armstrong W. (1994). On the difficulties of measuring oxygen release by root systems of wetland plants J Ecol 82 177-183

Stams A.J.M., Booltink H.W.G., Lutke-Schipholt I.J., Beemsterboer B., Woittiez J.R.W., Van Breemen N. (1991). A field study on the fate of ^{15}N-ammonium to demonstrate nitrification of atmospheric ammonium in an acid forest soil Biogeochemistry 13 241-255

Stefanson R.C. (1972) Soil denitrification in sealed soil-plant systems Plant Soil 33 113-127

Stenstrom M.K. and Poduska R.A. (1980) The effect of dissolved oxygen concentrations on nitrification Wat Res 14 643-649

Stephen J.R., McCaig A.E., Smith Z., Prosser J.I. and Embley T.M. (1996). Molecular diversity of soil and marine 16S rRNA gene sequences related to ß-subgroup ammonia-oxidizing bacteria Appl Environ Microbiol 62 4147-4154

Stephen J.R., Kowalchuk G.A., Bruns M.-A., Prosser J.I., Embley T.M. and Woldendorp J.W. Analysis of β subgroup chemolithotrophic ammonia oxidiser populations by DGGE, membrane transfer and hierarchical 16S rDNA oligonucleotide probing *in preparation*

Suzuki M.T. and Giovannoni S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR Appl Environ Microbiol 62 625-630

Teske A., Alm E., Regan J.M., Toze S., Rittmann B.E. and Stahl D.A. (1994) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria J Bacteriol 176 6623-6630

Teske A., Wawer C., Muyzer G. and Ramsing N.B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR amplified ribosomal DNA fragments Appl Eviron Microbiol 62 1405-1415

Tiedje J.M., Sextone A.J., Myrold D.D., Robinson J.A. (1982). Denitrification Ecological niches, competition and survival Antonie van Leeuwenhoek 48 569-583

Tiedje J.M. (1988) Ecology of denitrification and dissimilatoric nitrate reduction to ammonium In Biology of anaerobic microorganisms pp 179-244 Ed A J B Zehnder Wilder, New York

Tiedje J.M. (1994) Denitrifiers In Methods of soil analysis Part 2 Microbiological and biochemical properties pp 245-267 Eds R W Weaver, J S Angle and P S Bottomley Soil Science Society of America Inc , Madison

Troelstra S.R. (1983) Growth of *Plantago lanceolata* and *Plantago major* on a NO_3/NH_4 medium and the estimation of the utilization of nitrate and ammonium from ionic-balance aspects Plant Soil 70 183-197

Troelstra S.R., Wagenaar R., Smant W. and De Boer W. (1995) Soil nitrogen transformations and nitrate utilization by *Deschampsia flexuosa* (L) Trin at two contrasting heathland sites Plant Soil 176 81-93

Trofymow J.A., Coleman D.C. and Cambardella C. (1987) Rates of rhizodeposition and ammonium depletion in the rhizosphere of axenic oat roots Plant Soil 97 333-344

Turner S.M. and Newman E.I. (1984) Growth of bacteria on roots of grasses influence of mineral nutrient supply and interactions between species J Gen Microbiol 130 505-512

Uhel C., Roumet C., Salsac L. (1989). Inducible nitrate reductase of rice plants as a possible indicator of nitrification in waterlogged paddy soils Plant Soil 116 197-206

UtÂker J.B., Bakken L., Jiang Q.Q. and Nes I.F. (1996) Phylogenetic analysis of seven new isolates of ammonia-oxidising bacteria based on 16S rRNA gene sequences System Appl Microbiol 18 549-559

Verhagen F.J.M. and Laanbroek H.J. (1991). Competition for ammonium between nitrifying and heterotrophic bacteria in dual energy-limited chemostats Appl Environ Microbiol 57 3255-3263

Verhagen F.J.M., Duyts H. and Laanbroek H.J. (1993) Effects of grazing by flagellates on competition for ammonium between nitrifying and heterotrophic bacteria in soil columns Appl Environ Microbiol 59 2099-2106

Verhagen F.J.M., Hageman P.E.J, Woldendorp J.W. and Laanbroek H.J. (1994) Competition for ammonium between nitrifying bacteria and plant roots in soil in pots, effects of grazing by flaggelates and fertilization Soil Biol Biochem 26 89-96

Vlek P.L.G. and Byrnes B.H. (1986) The efficacy and loss of fertilizer N in lowland rice Fert Res 9 131-147

Voesenek L.A.C.J. (1990). Adaptations of *Rumex* in flooding gradients PhD thesis, University of Nijmegen

Voytek M.A. and Ward B.B. (1995) Detection of ammonium oxidising bacteria in the beta-subclass of the class Proteobacteria in aquatic samples with the PCR Appl Environ Microbiol 61 1444-1450

Wagner M., Rath G., Amann R., Koops H.-P. and Schleifer K.-H. (1995) *In situ* identification of ammonium oxidising bacteria Syst Appl Microbiol 18 251-264

Wagner M., Rath G., Koops H.P., Flood J. and Amann R. (1996) *In situ* analysis of nitrifying bacteria in sewage treatment plants Wat Sci Tech 34 237-244

Wagner R. (1994) The regulation of ribosomal RNA synthesis and bacterial cell growth Arch Microbiol 161 100-106

Ward D.M., Weller R. and Bateson M.M. (1990) 16S rRNA sequence reveal numerous uncultured microorganisms in a natural community Nature 345, 63-65

Watson S.W., Bock E., Harms H., Koops H-P., Hooper A.B. (1989). Nitrifying bacteria In Bergeys manual of systematic bacteriology, vol 3 pp 1808-1834 Eds J T Stacey, M T Bryant, N Pfennig and J G Holt Williams and Wilkins, Baltimore USA

Weisner S.E.B., Eriksson P.G., Granéli W. and Leonardson L. (1994). Influence of macrophytes on nitrate removal in wetlands Ambio 23 363-366

Werner W., Rey H.G. and Wielinger H. (1970) Uber die Eigenschaften eines neuen Chromogens fur die Blutzuckerbestimmung nach der GOD/POD-methode Z Anal Chem 252 224-228

Whipps J.M. (1984) Environmental factors affecting the loss of carbon from the roots of Wheat and Barley seedlings J Exp Bot 35 767-773

Whipps J.M. (1992) Carbon economy *In* The rhizosphere pp 59-97 Ed J M Lynch John Wiley and Sons, Chichester

White K. D. (1995) Enhancement of nitrogen removal in subsurface flow constructed wetlands employing a 2-stage configuration, an unsaturated zone, and recirculation Wat Sci Tech 32 59-67

Wittgren H.B. (1988) Removal of wastewater nitrogen in a wetland filter Linkoping studies in arts and sciences 29 (PhD thesis), Linkoping University, Sweden

Wittgren H.B. and Tobiason S. (1995). Nitrogen removal from pretreated wastewater in surface flow wetlands Wat Sci Tech 32 69-78

Woldendorp J.W. (1963) The influence of living plants on denitrification Meded Landbouwhogeschool, Wageningen 63 1-100

Woldendorp J.W. (1981). Nutrients in the rhizosphere In Agricultural yield potentials in continental climates Proceedings of the 16th colloqium of the international Potash institute, Warsaw 1981

Woldendorp J.W, Laanbroek H.J. (1989). Activity of nitrifiers in relation to nitrogen nutrition of plants in agricultural soils Plant Soil 115 217-228

Wood P.M. (1986). Nitrification as bacterial energy source In Nitrification Special publications of the society for general microbiology, vol 20 pp 39-61 Ed J I Prosser IRL Press, Oxford, UK

Yamanaka T. (1996). Mechanisms of oxidation of inorganic electron donors in autotrophic bacteria Plant Cell Physiol 37 569-574

Zar J.H. (1974) Biostatistical analysis Prentice-Hall Inc , Englewood Cliffs, USA

Zhang T.C., Fu Y.C. and Bishop P.L. (1994) Competition in biofilms Wat Sci Tech 29 263-270

Zumft W.G. (1992) The denitrifying prokaryotes In The prokaryotes pp 554-582 Eds A Balows, H G Truper, M Dworkin, W Harder and K-H Schleifer Springer Verlag, Berlin, Germany

Summary

An increased nitrogen input by agriculture and industry into terrestrial ecosystems over the last decades has led to environmental problems, especially in the Netherlands Although microbial nitrogen transformations (i e nitrification, denitrification) are known to be stimulated which results in acidification, elevated nitrate concentrations in sub-surface water, eutrophication of surface water and elevated emission of greenhouse gasses (NO and N_2O), insufficient information exists on the functioning and ecology of the bacteria involved in these processes This thesis aims to obtain information on the functioning of and interactions between nitrifiers and denitrifiers in the root zone of the oxygen-releasing emergent macrophyte *Glyceria maxima*

When the oxygen uptake kinetics of cells of the ammonia-oxidising bacterium *Nitrosomonas europaea* are compared with cells of the heterotrophic denitrifier *Pseudomonas chlororaphis* (Chapter 2), it appeared that nitrifiers are equally well equipped to function at low oxygen concentrations *N europaea* cells have higher oxygen uptake capacity in comparison to *P chlororaphis* cells, which compensates for the lower oxygen affinity of the nitrifier However, the low growth yield and metabolic efficiency of nitrifiers will not allow them to compete with heterotrophs for oxygen when sufficient carbon is available The oxidation kinetics of ammonium for *N europaea* and of glucose and glutamate for *P chlororaphis* suggest that the availability of electron donor will be a more important regulating factor than oxygen in the root zone of oxygen-releasing plants

To study possible interactions between nitrifiers and denitrifiers, gnotobiotic microcosms were developed, enabling pure culture studies in the root zone of the emergent macrophyte *G maxima* under controlled conditions By manipulating the photoperiod of the plant, important natural parameters (i e carbon and ammonia availability) could be varied in the root zone Elongation of the day length led to higher numbers of *P chlororaphis* This was due to a higher carbon exudation by *G maxima* (Chapter 3) With elongation of the day length, denitrification by this bacterium was also increased Inhibition of denitrification due to oxygen excretion could not be demonstrated The effect of the photoperiod clearly demonstrates the importance of the time in the growing season with respect to the effect of oxygen-releasing plants on denitrification

Using an identical experimental setup, population dynamics of the ammonia-oxidising *N europaea*, the nitrite-oxidising *Nitrobacter winogradskyi* and the denitrifier *P chlororaphis* were determined inside and outside the root zone of *G maxima* (Chapter 4) The presence of *G maxima* and an elongation of the day length again stimulated growth and denitrification activity of *P chlororaphis* However, growth of the nitrifiers was not affected by the presence of the plant Moreover, nitrification activity was suppressed when the day length was elevated This is due to ammonium limitation which is the result of uptake by the plant

When ammonium availability was not limiting for *N europaea*, the presence of *P chlororaphis* affected nitrification Potential nitrifying activities and growth of *N winogradskyi* were suppressed by the presence *P chlororaphis*, indicating the low competitive abilities of the nitrifiers

In a natural *G maxima* stand, nitrification and denitrification were stimulated in spring and early summer (Chapter 5) During the course of the growing season, numbers and activities of the nitrifying bacteria as well as denitrifying activities stayed at the same level in both the root zone and in the bare sediment Ammonium limitation due to plant uptake and oxygen limitation as a consequence of elevated heterotrophic activity are the most likely explanations for the suppression of nitrification during the growing season Comparison of the ammonia oxidising community from the root zone of *G maxima* with ammonia-oxidisers from a permanently oxic dune top revealed the presence of adaptive strategies to survive in anoxic or sub-oxic habitats The ammonia-oxidisers from the sediment were resistant to anoxic incubation and resumed nitrification activity immediately after the introduction of oxygen The specific oxygen affinities of the lake sediment community were high, enabling them to reach significant conversion rates at sub-oxic conditions

The dynamic pattern of numbers and activities and the adaptations of the ammonia oxidising community from the lake sediment could not be related to the community composition, as determined with molecular biological techniques (Chapter 6) Inside and outside the root zone of *G maxima,* the species composition of the ammonia-oxidising community was characterised exclusively by the presence of 16S rDNA sequences typical for the genus *Nitrosospira Nitrosomonas* related sequences could only be detected upon enrichment The comparison of ammonia-oxidising communities of one dune and two grassland soils with that of the lake sediment, did not provide any evidence for a phylogenetically distinct group responsible for the observed differences in the tolerance to anoxic conditions and in K_m for O_2 This suggests that the observed adaptations have a physiological origin rather than being the properties of a specific ammonia oxidising species, which is characteristic for a particular environment

This thesis demonstrates that the occurrence of nitrification in the root zone of oxygen-releasing plants is entirely dependent on the plant and on the activity of other oxygen- and ammonia-consuming bacteria, which will strongly depend on the time of the year When conditions permit nitrification, the nitrifying community is very well equipped to anticipate and to profit from the opportunity Prolonged survival without oxygen and high specific oxygen affinity enable nitrifiers to function in this habitat The strategy of longevity and stress tolerance will also be of advantage for survival in other habitats

Samenvatting

De hoge stikstofdepositie door landbouw en industrie gedurende de laatste decennia heeft in Nederland tot milieuproblemen geleid. Door deze hoge beschikbaarheid van stikstof in terrestrische oecosystemen wordt met name de omzetting van ammonium naar nitraat (nitrificatie) en de reductie van nitraat tot gasvormig N_2, N_2O of NO (denitrificatie) gestimuleerd. Deze processen zorgen voor verzuring van de bodem, verhoging van de nitraatconcentratie in drinkwater, eutrofiëring van oppervlaktewater en voor een verhoogde emissie van de broeikasgassen NO and N_2O. Over het functioneren en de ecologische karakteristieken van de betrokken bacteriën in natuurlijke systemen is relatief weinig bekend. Dit proefschrift heeft tot doel informatie te verkrijgen over de interacties tussen nitrificerende en denitrificerende bacteriën door ze te bestuderen in de wortelomgeving van de emergente macrofyt *Glyceria maxima* (Liesgras). De wortels van deze plant verliezen zuurstof aan het omringende sediment hetgeen van grote invloed kan zijn op de bacteriële processen in de wortelzône.

Uit de vergelijking van de zuurstof opnamekinetiek van de ammonium-oxydeerder *Nitrosomonas europaea* met die van de denitrificeerder *Pseudomonas chlororaphis*, blijkt de ammonium-oxydeerder evengoed uitgerust te zijn voor het functioneren onder lage zuurstofspanning als de denitrificeerder (Hoofdstuk 2). De lagere affiniteit voor zuurstof van *N. europaea* wordt gecompenseerd door de hogere omzettingscapaciteit per cel (V_{max}). Wanneer er echter voldoende substraat voor de denitrificeerder aanwezig is, zal de nitrificeerder niet kunnen concurreren om zuurstof door het lage metabole rendement en de daarmee gepaard gaande lage biomassaproductie. Vergelijking van de oxydatiekinetiek van de electrondonor voor *N. europaea* en *P. chlororaphis* leidt tot de conclusie dat de beschikbaarheid van ammonium and organisch koolstof waarschijnlijk belangrijkere regulerende factoren zijn voor de interacties tussen nitrificeerders en denitrificeerders dan zuurstof.

Om de mogelijke interacties tussen nitrificerende en denitrificerende bacteriën te bestuderen werden speciaal ontwikkelde, gnotobiotische microcosmossystemen gebruikt, die het mogelijk maakten om onder gecontroleerde omstandigheden experimenten met reincultures uit te voeren, in de aanwezigheid van de emergente macrofyt *G. maxima*. Door de lichtperiode van de plant te manipuleren was het mogelijk belangrijke natuurlijke parameters (zuurstof- en ammoniumbeschikbaarheid) in de wortelzône te variëren. Het verlengen van de lichtperiode resulteerde in hogere aantallen van de denitrificeerder *P. chlororaphis* ten gevolge van de hogere uitscheiding van organisch koolstofverbindingen door *G. maxima* (Hoofdstuk 3). De denitrificatie-activiteit van *P. chlororaphis* werd eveneens gestimuleerd door het verlengen van de lichtperiode van *G. maxima*. Er waren geen aanwijzingen voor een eventuele remming van de denitrificatie-activiteit door zuurstof afkomstig van *G. maxima*. Het effect van de daglengte op denitrificatie in de wortelzône van *G. maxima* geeft duidelijk het belang van de tijd

in het groeiseizoen weer als regulerende factor
De populatiedynamica van de ammonium-oxydeerder *N europaea*, de nitriet-oxydeerder *Nitrobacter winogradskyi* en de denitrificeerder *P chlororaphis* werd in gnotobiotische systemen bepaald binnen en buiten de wortelzône van *G maxima* (Hoofdstuk 4) De aanwezigheid van de plant en het verlengen van de lichtperiode stimuleerden opnieuw zowel de groei van *P chlororaphis* als de denitrificatie door deze bacterie De groei van de nitrificeerders werd niet beinvloed door de plant De nitrificatie-activiteit werd zelfs onderdrukt door het verlengen van de lichtperiode omdat ten gevolge van opname door de plant ammoniumlimitatie optrad Wanneer ammonium voldoende beschikbaar was voor *N europaea* werd de potentiele nitrificatie-activiteit en de groei van *N winogradskyi* onderdrukt door de aanwezigheid van *P chlororaphis* Dit duidt op de slechte competitieve eigenschappen van nitrificerende bacterien
Onder natuurlijke omstandigheden werd zowel nitrificatie als denitrificatie bevorderd door de aanwezigheid van *G maxima* (Hoofdstuk 5) Deze stimulatie werd echter alleen in het voorjaar en de vroege zomer waargenomen In het verdere verloop van het groeiseizoen was er geen sprake meer van verhoogde nitrificatie- en denitrificatie-activiteit in de wortelzône Ammoniumlimitatie ten gevolge van een verhoogde opname door de plant is hiervoor een mogelijke verklaring, net als zuurstof-limitatie door een stimulatie van de heterotrofe microbiota Een combinatie van beide is ook mogelijk als verklaring voor de onderdrukking van de nitrificatie en de daaraan gekoppelde denitrificatie De ammonium-oxydeerders uit de wortelzône van *G maxima* bleken aanpassingen te vertonen aan het leven in de anoxysche/suboxysche habitat in vergelijking met ammonium-oxydeerders van een permanent oxysche duinlocatie Incubatie onder anoxysche omstandigheden had geen invloed op de ammonium-oxydeerders uit de wortelzône van *G maxima* Deze bacterien hervatten namelijk onmiddellijk hun activiteit wanneer zuurstof werd toegevoegd, terwijl de ammonium-oxydeerders uit de duinen pas na een aantal uren weer actief werden, en wel op een niveau dat veel lager was dan zonder anoxysche incubatie Bovendien bezitten de ammonium-oxydeerders uit de wortelzône van *G maxima* een zeer hoge specifieke affiniteit voor zuurstof waardoor ze zeer goed in staat zijn bij lage zuurstofspanningen te functioneren
De seizoensdynamiek en de gevonden aanpassingen konden niet teruggevoerd worden tot veranderingen in de soortensamenstelling van de ammonium oxyderende gemeenschap Een moleculair-biologische analyse toonde aan dat zowel binnen als buiten de wortelzône van *G maxima* alleen *Nitrosospira*-achtige soorten voorkomen *Nitrosomonas*-achtige soorten konden alleen in ophopingen worden aangetoond De vergelijking van de ammonium-oxyderende gemeenschap van een duinlocatie en van twee graslanden met die uit de wortelzône van *G maxima*, leverde geen aanwijzingen voor de aanwezigheid van een specifieke fylogenetische groep die verantwoordelijk zou kunnen zijn voor de gevonden verschillen ten aanzien van de mate van tolerantie ten opzichte van anoxysche omstandigheden en

affiniteit voor O_2 Dit leidt tot de conclusie dat de aanpassingen waarschijnlijk een fysiologische basis hebben en niet het gevolg zijn van de aanwezigheid van een specifieke soort ammonium oxydeerder in de wortelzône van *G maxima*
Dit proefschrift laat duidelijk zien dat het optreden van nitrificatie in de wortelzône van planten die zuurstof uitscheiden volledig afhankelijk is van de plant en de activiteit van andere, met name heterotrofe, bacterien, hetgeen weer zeer sterk afhankelijk is van de tijd in het groeiseizoen Nitrificeerders zijn zeer goed uitgerust om snel te anticiperen op en te profiteren van "goede" condities Het overleven van langdurige anoxysche periodes en het bezitten van een zeer hoge specifieke affiniteit voor zuurstof maakt deze organismen uiterst geschikt om te functioneren in de wortelzône van planten die zuurstof uitscheiden De "lange adem" strategie en de mate van stress-tolerantie is wellicht van voordeel bij overleving in diverse habitats

List of scientific publications

Full length papers

Engelaar W.M.H.G., Bodelier P.L.E., Laanbroek H.J. and Blom C.W.P.M. (1991) Nitrification in the rhizosphere of a flooding-resistant and a flooding non-resistant *Rumex* species under drained and waterlogged conditions FEMS Microbiol Ecol 86 33-42

Samecka-Cymerman A., Kempers A.J. and Bodelier P.L.E. (1991) Preliminary investigations into the background levels of various metals and Boron in the aquatic liverwort *Scapania uliginosa (Sw) Dum* Aquat Bot 39 345-352

Weekers P.H.H., Bodelier P.L.E., Wijen J.P.H. and Vogels G.D. (1993) Effects of Grazing by the Free-living soil amoeba *Acanthamoeba castellanii, Acanthamoeba polyphaga,* and *Hartmanella vermiformis* on various bacteria Appl Environ Microbiol 59 2317-2319

Laanbroek H.J., Bodelier P.L.E. and Gerards S. (1994) Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* grown in mixed continuous culture at different oxygen concentrations Arch Microbiol 161 156-162

Bodelier P.L.E., Libochant J.A., Blom C.W.P.M. and Laanbroek H.J. (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low oxygen or anoxic habitats Appl Environ Microbiol 62 4100-4107

Bodelier P.L.E., Wijlhuizen A.G., Blom C.W.P.M. and Laanbroek H.J. (1997) Effects of photoperiod on growth of and denitrification by *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima*, studied by means of a gnotobiotic microcosm Plant Soil 190 91-103

Bodelier P.L.E. and Laanbroek H.J. (1997) Oxygen uptake kinetics of *Pseudomonas chlororaphis* grown in glucose- or glutamate-limited continuous cultures Arch Microbiol 167 392-395

Bodelier P.L.E., Duyts H., Blom C.W.P.M. and Laanbroek H.J. Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima* Submitted with FEMS Microbiol Ecol

Kowalchuk G.A., Bodelier P.L.E., Heilig G.H.J., Stephen J.R. and Laanbroek H.J. Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and sediments, using PCR, DGGE and oligonucleotide probe hybridization In preparation for FEMS Microbiol Ecol

Abstracts

Laanbroek H.J., Gerards S. and Bodelier P.L.E. (1992) Kinetics of oxygen consumption by *Nitrosomonas europaea* and *Nitrobacter agilis* grown in energy-limited chemostats at different oxygen tensions Poster abstract In Abstracts of the 6th International Symposium on Microbial Ecology, Barcelona, Spain

Bodelier P.L.E., Blom C.W.P.M. and Laanbroek H.J. (1995) Effects of the emergent macrophyte *Glyceria maxima* on nitrification and denitrification Abstract of oral communication In Abstracts of the 7th International Symposium on Microbial Ecology, Santos, Brazil

Curriculum vitae

De auteur van dit proefschrift, Paul Bodelier, werd geboren op 16 december 1966 te Kerkrade. In deze plaats volgde hij het basisonderwijs van 1973 tot 1979. In de periode 1979-1985 doorliep hij het VWO aan het Sancta Maria College te Kerkrade. In 1985 begon hij met de biologiestudie aan de Katholieke Universiteit Nijmegen. Tijdens deze studie werden twee hoofdvakken en een bijvak gevolgd. Tijdens de eerste hoofdrichtingsstage, Experimentele Plantenoecologie, werden de effecten van inundatie op de stikstofvoeding van *Rumex* soorten en nitrificerende bacteriën in hun rhizosfeer onderzocht. Deze stage werd uitgevoerd op de toenmalige afdeling Bodembiologie van het Instituut voor Oecologisch onderzoek. De tweede hoofdrichtingsstage werd uitgevoerd op de afdeling Microbiologie en Evolutiebiologie van de KUN. Tijdens deze stage stonden de kwantificatie van celgroei en stikstofmineralisatie door bodemamoeben in monoxene culturen centraal. In het kader van een bijvak werd onderzoek gedaan naar biomonitoring van zware metalen met behulp van sub-aquatische mossen. Deze stage werd uitgevoerd op de afdeling Aquatische Oecologie en Biogeologie van de KUN. Het doctoraal examen werd met goed gevolg afgelegd in 1991. Aansluitend trad hij in dienst van de Koninklijke Nederlandse Academie van Wetenschappen (KNAW) en werkte als onderzoeksassistent op de afdeling Bodembiologie van het toenmalige Instituut voor Oecologisch Onderzoek te Heteren. Hij onderzocht hier de mogelijkheid om de nitrificerende bacterie *Nitrosospira AHB1* op grote schaal op te kweken. Deze aanstelling ging over in een positie als Onderzoeker in Opleiding in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) voor de periode van 1992 tot 1996. In deze periode werd het in dit proefschrift beschreven onderzoek uitgevoerd bij de afdeling Plant-Microorganisme Interacties van het Centrum voor Terrestrische Oecologie van het Nederlands Instituut voor Oecologisch Onderzoek te Heteren. Tevens werd in deze periode de landelijke AIO/OIO opleiding van de Onderzoeksschool Functionele Ecologie gevolgd en met goed gevolg afgelegd. Vanaf 1 mei 1997 is de auteur van dit proefschrift als post-doc medewerker verbonden aan het Max-Planck Institut für Terrestrische Mikrobiologie in Marburg, Duitsland. Tijdens deze aanstelling zullen de interacties tussen ammonium- en methaan-oxyderende bacteriën in de rhizosfeer van rijst onderzocht worden.

Paul Bodelier is getrouwd met Monika Bade. Zij hebben een dochter Iris en een zoon Mathijs.